AVIAN LEUKOSIS
CONFERENCE, East Lansing, MI

April 25--26, 1962

Regional Poultry Research Laboratory

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U. S. Department of Agriculture,
Agricultural Research Service,
Animal Husbandry Research Division,
Regional Poultry Research Laboratory //
5a East Lansing, Michigan

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PREFACE

The Centennial celebration of the U. S. Department of Agriculture and the Land-Grant Colleges seemed an appropriate time to stimulate renewed interest in research on the Avian Leukosis Complex.

The Avian Leukosis Conference was called to permit an exchange of knowledge and to stimulate new approaches among scientists. As these proceedings will show, much has been learned about "Avian Leukosis", many new tools have been developed, and we look with renewed confidence for important new breakthroughs in conquering the disease.

It is believed that this Conference and others that may follow will help build the momentum for an ever increasing tempo of attack on the many problems to be solved. The prospects for success certainly seem brighter than at any time in the past. Success will be beneficial not only to the poultry industry, but hopefully to mankind as well in pointing the way in his struggle to conquer cancer.

Steven C. King
Chief, Poultry Research Branch
Animal Husbandry Research Division

AVIAN LEUKOSIS CONFERENCE

Berley Winton, Director of the Regional Poultry Research Laboratory, presided at the Wednesday morning session, April 25. He welcomed those in attendance on behalf of the Laboratory and introduced representatives of the United States Department of Agriculture and of Michigan State University.

He announced that a roster of those in attendance would be compiled and mailed to each one attending the conference, together with a copy of the report of conference proceedings and a bibliography of published papers at the Regional Poultry Research Laboratory.

Dr. Howard Johnson, Director, Animal Disease and Parasite Division, ARS, USDA, presided at the Wednesday afternoon session.

Dr. R. E. Hodgson, Director of the Animal Husbandry Research Division, ARS, USDA, presided at the dinner meeting Wednesday evening.

Thursday morning's session, April 26, was presided over by Dr. S. C. King, Chief, Poultry Research Branch, Animal Husbandry Research Division, USDA. After intermission it was decided to continue with the talks scheduled for the afternoon without a break for lunch in order to give more time for the tour of the Regional Poultry Research Laboratory, and yet allow certain registrants to make plane schedules.

Reports as available will follow in the order in which they were presented on the program.

Avian Leukosis - an Enigma for Research Scientists

T. C. Byerly[1]/

My first scientific interest in the problem of neoplasms occurred during my research toward a PhD. At that time, I found that unorganized growth continued after cessation of organized growth caused by exclusion of air from early chick embryos. It occurred to me that such growth must depend on glycolysis rather than oxidation. The idea was perfectly sound but not as new as I soon discovered in reviewing the literature that Warburg had demonstrated glycolysis as a principle energy source in the young chick embryo and in neoplastic cells. I did a great many hours of research on mitosis in the embryo and factors regulating it during the following ten years. I found, as have many others, that mitosis in the chick embryo follow regular patterns in time and place, conditioned by genetic capacity, internal and external environment.

My first experience with the avian leukosis complex was at Beltsville in 1932. The disease devastated the flocks there that year--flocks that in the previous several years had suffered minimal laying house mortality. The disease continued to be a major source of mortality at Beltsville as in the United States generally. The RPL was set up in 1938, the first year of my stay at the University of Maryland. Hal Devolt and I cooperated in a limited series of research studies there as cooperative agents of RPL. The avian leukosis complex overwhelmed control as well as experimental lots. Since that time I have followed research at RPL with interest and on occasion with justified enthusiasm.

Let us examine the record of achievement. I shall not attempt to recite it. Others will emphasize achievements that I omit. I shall add some assumptions which seem to me to be reasonable and compatible with experiment and experience here and at other laboratories and in the field. The word enigma in the title of my talk is obviously appropriate.

Established facts seem to me to include the following. The role of this laboratory in their establishment has been major but not exclusive. First, there is a genetic basis for resistance and susceptibility to the expression of the disease. Waters and his colleagues at RPL and Hutt and Cole at Cornell and others have amply demonstrated this fact. Second, both infectious agent and passive immunity may be transmitted through the egg. I state this as established fact knowing that some may

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1/ Deputy Administrator, Farm Research, United States Department of Agriculture

consider the first portion of the proposition as trivial if true at all. Third, there is a transmissible agent, a virus, associated with the disease. Fourth, severity of the disease, and even its cytopathology, are a function of dosage of the transmissible agent when experimentally applied. Fifth, infection of the embryo or very young chick results in earlier and generally more severe disease response than later infection. Sixth, variability of expression of the disease in the same and different genetic stocks in time and place is extreme. Seventh, the disease behaves as a malignant neoplasm.

These seven points are by no means a complete catalogue.

It is perhaps mere description, and certainly not new to consider that the initiation of a neoplasm in an individual depends on the loss by a cell or cells of its tissue-specific pattern so that such cells fail to be recognized by the growth-regulating mechanisms and proliferate unchecked. Does the virus of avian leukosis behave thus as a mutagen? And if so, under what circumstances? Let us assume for purposes of discussion that all mutagens are carcinogens, that the avian leukosis agent is a virus and a mutagen-carcinogen. What then is peculiar about this virus that it should behave as a mutagen and not other avian viruses--e.g., Newcastle, fowl-pox, ornithosis? Is it possible that association of host DNA and virus RNA causes systematic change in host DNA analogous to the transfer of genes from one bacterial population to another via DNA? This hypothesis might be more hopeful than it now appears to be had Shoffner's blood transfusion experiments yielded positive results and confirmed the reported success of Soviet scientists.

In any case, it is reasonable to assume genetic change in the neoplastic cells though perhaps not necessary to make this assumption. What is the genetic basis that makes the postulated genetic change highly probable in infected genetically susceptible birds, much less probable in genetically resistant birds? Studies of intermediary metabolism provide opportunities for identification of genetic change. There is much evidence as well as sound reason for the validity of the one gene-one enzyme hypothesis.

If genetic change in response to infection with the agent of the avian leukosis complex or if the genetic difference between resistant and susceptible birds rests on one or only a few genes, it should be possible to identify the enzyme or enzymes affected. Plapp at the Corvallis Laboratory of the Entomology Research Division has identified a substantial difference in ali esterase in the housefly resistant to malathion and consequently found that certain phosphorous compounds synergized the insecticides and made them effective again.

Anti metabolletes, substances which contain certain groups of atoms in their molecules capable of forming a complex with an enzyme or analogously with a purine, pyrimidine or animo acid are under intensive and extensive laboratory and clinical research with respect to neoplasms.

Let us return to the immunological area. What information can we gain from auto immunological reactions? Is it possible to immunize against infective nucleric acid? Or to find chemicals which may inactivate such infective nucleic acid without destroying the antigenic capacity of the associated protein? Will it ever be possible to render an animal once infected with a small particle virus free of that virus--or to render its semen or other secreta and excreta truly free? Heat readily inactivates the whole virus but it takes a lot of it to destroy nucleic acid.

While experience indicates small or absent immunogenic response on the part of embryo or newly hatched chick, what of interferon?

Before we leave the host-parasite relationship, let us consider for a moment its bases. In flax-flax rust relationships, Flor of the CRD stationed at North Dakota Agricultural Experiment Station has established a gene-for-gene relationship between host and parasite. Flor and his colleagues have established the presence of a trace of a common antigen in susceptible host and virulent rust. What is the chemical relationship between susceptible chicken DNA and avian leukosis RNA? Is it yet established that this and other chicken viruses are not just chicken RNA running wild? Is there a virus which can reproduce outside a living host cell?

This seminar will summarize and evaluate the existing information on genetics, immunology and virology and pathology relevant to the avian leukosis complex. These are proper and highly productive research areas. Facilities are yet lacking for adequate exploration of all the possible ecological factors and their possible interactions--certainly with respect to a complete appraisal of the external environment and the expression of the disease. The current epizootiological study may help.

With respect to the internal environmental factors, no such limitation is apparent. Studies of radiation have not thus far been highly productive. Hormonal studies have not yet contributed outstanding results. Nutritional studies are really only beginning. It is a common place that animals fed a luxus diet (ad lib) show a higher incidence of neoplasms generally speaking than similar animals fed the same diet in restricted amounts. There are some fairly extensive studies of feed restrictions with chickens; have the results no relevance? or are the data inadequate? What specific nutrients or toxicants are relevant?

The role of hormones, if any, in the expression of leukosis is uncertain. My own research indicating lower laying house mortality in broody than in non-broody birds has been confirmed by Hutt. The frequent association of the onset of leukosis mortality with the initiation of egg production may be only coincidental. There is sufficient research on the relation of hormones to the disease. Work is needed not only on the obvious estrogens and androgens, but on pituitary, adrenal, thyroid, gonadal, and other hormones as well. Their interaction with genes, virus and with other environmental factors is unknown. Until Burmester and his colleagues established a technique for quantitative assay of the infective agent, precise studies of interaction were impossible. They are still difficult.

One basic difficulty lies in the non-availability of stock certainly free of the avian leukosis virus. When facilities become available for adequate segregation of enough populations, perhaps such stocks can be established.

Enigma? How has it been possible for the staff here and for their colleagues in other laboratories to make the solid achievements they have made with so complex a problem?

The Avian Leukosis Problem in Ohio

Glyde A. Marsh[1]/

During the past year, the official state diagnostic laboratory reported 180 cases of avian leukosis in approximately 1615 poultry cases. However, these figures are of little value in evaluating the importance of this disease.

Few cases of leukosis reach laboratories. Most servicemen and poultrymen feel competent to diagnose the condition on the basis of gross signs. Commercial people tend to hide problem flocks. Trade rumors of leukosis problems have an adverse effect on the sale of chicks and pullets. Badly infected flocks tend to be slaughtered at plants not maintaining inspection. Although the figures don't show it, leukosis is still the No. 1 disease in our state.

In the last few years, the pattern of the disease is changing. I am seeing less of the ocular form, very little neural, more osteopetrosis, less of the leukemic forms. More visceral leukosis is apparent and the pattern is changing. It is not unusual to observe visceral tumors in pullets only 6 or 7 weeks of age.

I see little evidence of differences in resistance by commercial strains. One franchise group seems to have a problem today. Next year this stock is doing well and someone else is in trouble. They all seem to have their turn.

Leukosis does not remain a problem on farms where the breeding stock is kept closed. For what they are worth, I would like to present these field observations. Mixing populations of chicks from several sources promotes the problem. Some influences in the growing period increase the number of clinical cases observed when the birds have matured.

Like many others working in the field, I am very much confused by the fact that this disease hits well managed flocks. Many puzzling cases are observed that are not explainable by our present research results. An example is a case where some 6500 birds were grown in a new house. At sixteen weeks, half of the flock was transferred to another new building. In a few weeks heavy losses from visceral leukosis were experienced. The remaining birds were housed in several smaller flocks on other farms. In them no losses were seen.

The possibility exists that some simple solution may be available to the industry to permit it to live with the disease. For this reason, a field study is being planned for Ohio.

1/ Extension Specialist, Ohio State University, Columbus, Ohio

The Avian Leukosis Problem in Processing Plants

Frank E. Blood[1]/

Paragraph 81.81 of the Regulations Governing the Inspection of Poultry and Poultry Products, provides that carcasses of poultry affected with any one or more of the several forms of avian leukosis complex shall be condemned, except that carcasses affected with ocular form only may be passed (the ocular form is relatively minor and has been observed only in mature chickens).

During the past three years, we have observed avian leukosis on all types and classes of poultry with the exception of ducks.

The incidence of leukosis as observed on post mortem inspection is much higher in mature chickens than in any other class or type of poultry. However, the incidence of leukosis in broiler-fryers and fryer-turkeys is increasing at such a rate that we feel this fact should be emphasized.

YOUNG CHICKENS

United States - 1959-61

Year	Head Inspected	% Condemned Post-Mortem	Head Condemned for Leukosis	Leukosis as a % of Total Head Condemned
	(Mil.)		(Mil.)	
1959	1,415	1.47	1.0	4.6
1961	1,726	1.94	1.9	5.8

1 Deputy Director, Poultry Division, Agricultural Marketing Service

YOUNG CHICKENS

By Area - 1959-61

Area	Head Inspected[1] (Thou.)	Head Condemned for Leukosis (Thou.)	Head Inspected (Thou.)	Head Condemned for Leukosis (Thou.)
Philadelphia	366,410	409.8	390,316	871.7
Chicago	107,830	42.5	80,549	44.7
Des Moines	9,966	2.9	23,452	12.5
San Francisco	39,793	12.9	49,014	21.1
Atlanta	652,478	380.6	824,014	595.6
Dallas	238,595	153.1	358,762	352.2

As the husbandry and management practices in the turkey industry adjust to a closer similarity with established practices in the broiler-fryer industry, the manifestation and incidence of leukosis of turkeys more closely resembles those noted in the broiler-fryer operations. For instance, until three years ago, we did not recognize types of leukosis in turkeys other than visceral lymphomatosis; today all types are being observed on our inspection lines. This has become important to the poultry industry since our regulations provide that all birds showing leukosis lesions, except ocular, must be condemned.

With the initiation of mandatory inspection on January 1, 1959, we intensified our efforts nationwide to develop and cultivate a closer working relationship between local, state and Federal Disease Eradication and Control officials and our folks engaged in poultry inspection. Since we are looking at vast numbers of birds every day--of all classes--and with all degrees of pathological conditions, we felt that we were in the best position to alert the various agencies as to the existence of disease conditions and any changes noted in either incidence or manifestations. Because of the speed of operations in a processing plant, our inspectors must operate on the basis of the manifestations of disease conditions in poultry and do not have the time necessary for diagnostic analysis. We feel that when we encounter particularly acute disease conditions in a flock or plant

1 Revised 4/24/62

and can make on the spot referrals to those in a position to do something about it, diagnosis and corrective follow-up measures can be instigated at the best possible moment. Here I want to cite two examples; first, in the past year a study and identification of histomonas meleagridis in turkeys and its various manifestations was undertaken by members of a team from ARS and AMS. Because of the close resemblance of leukosis and atypical cases of blackhead, finer lines of demarcation and identification were needed for guidance of on-the-line inspectors. As a result of the efforts of this study team, we feel that the two conditions can now be separated with accuracy. Second, a producer in Nashville, Arkansas, encountered a serious poultry disease outbreak which was causing heavy death losses in his production flocks and heavy condemnations at processing plants. This producer requested assistance of our area office in Dallas. The request was relayed to our Washington officials who in turn contacted ADE officials of ARS. Dr. Mulhern contacted Dr. Walker in Nashville, Tennessee, who immediately proceeded to Arkansas on this matter. The fact that the original call for assistance was made on a Wednesday and by that Friday disease control and eradication officials were operating on the problem in Arkansas, has created quite a bit of admiration for the efforts and abilities of our Department.

In conclusion, let me say that we in the poultry inspection field feel that by assuring the American public that it is receiving wholesome poultry and by pointing out disease conditions to those with the authority and ability to eradicate or control such conditions, that we are doing the entire poultry industry a valuable service. We certainly appreciate the wholehearted cooperation and assistance which we are receiving in accomplishing this mission.

Transmission of Tumor Viruses Isolated from Field Cases of the Avian Leukosis Complex

T. N. Fredrickson

Transmission of visceral lymphomatosis has been attempted by many workers in order that the disease might be studied in the same logical way that most other infectious diseases have been treated. Most of those who tried to passage visceral lymphomatosis were unsuccessful, and reports of success were generally treated with scepticism. Material for transmission consisted of either implants of cellular components of lymphoid tumors from commercially raised chickens or filtered extracts of such tumors. Olson and Pentimalli were able to propagate lymphoid tumors using the former method, and Furth transmitted visceral lymphomatosis as well as other diseases of the avian leukosis complex with filtered extracts of spontaneously occurring lymphoid tumors. As work began at the Regional Poultry Research Laboratory, it was evident that in order to study the disease, quantities of virus which would induce the disease with a quantitative and qualitative response would be required. Accordingly, both cellular and later cell-free material was tested in susceptible chickens for oncogenic potency. It was found that cells, and in some cases extracts of lymphoid tumors from chickens in the genetic flock at the Laboratory, induced high incidences of visceral lymphomatosis, erythroblastosis and, to a much lesser degree, osteopetrosis and hemangeomatosis. In addition, it was found that filtrates of a transmissible lymphoid tumor passaged many times with cellular material by Olson were potent sources of tumor virus. The virus from Olson's tumor was characterized both quantitatively and qualitatively and is now referred to as RPL 12. Most of the research work on leukosis at the Regional Poultry Research Laboratory during the last decade has been done with the virus of RPL 12. A continual question in the minds of those engaged in research on the fowl tumors has been on the relationship of the fowl tumor viruses affecting chickens in the field to RPL 12 virus. It appeared necessary, therefore, to obtain tumor material from chickens in the field and to attempt to passage virus from these tumors. The material was inoculated into L15I birds since they were known to have a very low incidence of tumors of any kind although they were highly susceptible to the disease.

Tumor tissue was collected from 22 different sources in the Northeast, Southwest, Midwest, and West Coast areas of the U. S. A. The principal type of leukosis involving the flocks from which donor birds were selected was visceral lymphomatosis (20 flocks). In addition, cases of neural lymphomatosis (1 flock), ocular lymphomatosis (1 flock), and nephroblastoma (1 flock) were also taken for transmission studies. Chickens from several types of flocks were among those providing donor material including commercial egg, breeders for hatching eggs, broilers, and those raised for experimental purposes.

The age of the chickens in these flocks varied from 10 weeks to 16 months with a median of 5 1/2 months. All but two of the eee visceral lymphomatosis lesions were judged, on the basis of histopathology, to be highly malignant. There was extensive replacement of the normal tissues with large anaplastic lymphoid tumor cells among which many mitotic figures were evident. In the two tumors which appeared less malignant, the tumor cells were composed chiefly of medium and small lymphocytes and considerable fibrous connective tissue proliferation was seen in one of the tumorous livers. Some of the neural lymphomatosis lesions were composed of large, obviously malignant lymphoid tumor cells, while in others predominantly small lymphocytes and some plasma cells were seen. The ciliary body, in the cases of ocular lymphomatosis, was infiltrated with medium and small sized lymphocytes and some plasma cells. The one renal adenoma used as a source of transmission material was made up of very large neoplastic epithelial cells which formed papillary cysts; the solitary tumor was completely walled off from the surrounding normal renal parenchyma.

The tumor tissue which was later used for transmission was collected immediately after the donor bird had been necropsied. Small pieces of tumorous organs were placed on glass tubes and frozen in solid carbon dioxide. A portion of each tumor which was later used was checked to ensure that it contained no other infectious agents. Extracts for transmission was prepared by placing a weighed amount of tumor in Simms' salt solution to make a five percent w/v solution. The solid tumor was then homogenated with a Virtus grinder so that complete cell disruption had taken place. This homogenate was then centrifuged at 1,200 G for 30 minutes. The supernate was pipetted off. Usually the supernate was inoculated but occasionally it was recentrifuged at 30,000 G for 60 minutes to obtain a high speed pellet which was resuspended in Simms' salt solution and then inoculated. No real difference was noted in the types and numbers of neoplasms induced by the high speed pellet as compared with the low speed supernate. Day-old chicks inoculated by the intraperitoneal route were used for the first two passages of each strain. In later passages 2-week old chicks inoculated intravenously were used. This change in the ages of the inoculated chicks was an attempt to avoid high incidences of osteopetrosis. All chickens inoculated with extracts from a single source were housed in pens isolated from those containing chickens inoculated with material from different sources. Attendants caring for the chickens had to change their clothes before entering any of these semi-isolated pens.

Results

Many different breeds and strains of chickens supplied the donor material. Ages of the chickens in the donor flocks varied from 2 1/2 months to 15 months with the median age of 5 1/2 months. In many

of the donor flocks, especially those in the eastern part of the country, a high incidence of visceral lymphomatosis ranging from ten to thirty percent had been observed within a period of 10 weeks or less. Mortality of this kind from lymphomatosis seemed to follow a rather distinct pattern and we have referred to it as "acute lymphomatosis." Most flocks affected by acute lymphomatosis undergo this high mortality before they are 20 weeks of age, after which the low, continuous, unspectacular mortality usually associated with lymphomatosis ensues for the rest of the laying period. Material was obtained from these outbreaks of acute lymphomatosis in order to see if a similar disease pattern could be induced in inoculated L15I chickens. In addition, material was also obtained from older flocks in which a high incidence of lymphomatosis had been noted over periods of several months. In other flocks, namely three from California and that from Albion, Michigan, the incidence of lymphomatosis could not be considered much above that observed in most layer operations.

Microscopic examination was made of tissues which were used for transmission studies.

In most cases materials from several individual chickens were used for the initial transmission of the field virus. There was some variation in the response among the various inocula from one source but in those sources giving a high initial response very few inocula proved to be low in virus content. The variation among flocks was shown to be significantly greater than the within-flock variation by the F test. Of the 19 sources of visceral lymphomatosis tumors, nine induced neoplastic mortality in the recipient L15I chickens of more than fifty percent. This is to be compared to a zero percent incidence of tumors in the uninoculated controls. By far the majority of the neoplasms were visceral lymphomatosis but considerable erythroblastosis and osteopetrosis were also induced. Of the 10 flocks with a low response, 4 were from older hens from the flocks in which the incidence of lymphomatosis in the flocks was relatively low. In 6 other flocks, however, there was a high incidence and the disease pattern was of the acute type. There was no correlation between the malignancy index of the tumors extracted for transmission and their content of potent virus. No particular tissue was a better source of virus than others used and thus, liver, spleen, heart, bursa, and kidney all induced high neoplastic responses. Skeletal muscle was the one tissue which gave a uniformly low response.

Both neural and ocular lymphomatosis gave a low response principally of visceral lymphomatosis. On the other hand, the nephroblastoma tissue induced a high initial response of visceral lymphomatosis. Although initial results were suggestive of low content of potent virus in the neural and ocular material, a subsequent second passage of visceral lymphomatosis extracts from tumors induced in the first

passage of visceral lymphomatosis were highly potent, inducing up to a hundred percent mortality from visceral lymphomatosis. No ocular, and very infrequently neural lymphomatosis, was observed in all the transmission studies.

The inocula giving a high initial response were further passaged in L15I chickens. An attempt was made to choose donors from inoculation lots with a uniformly high neoplasm incidence. Thus, some series ended in favor of carrying on others. Both visceral lymphomatosis and erythroblastosis lesions were used in the ensuing passages, the former in order to keep a straight line of visceral lymphomatosis and the latter to try to increase the oncogenic potency for that particular strain of virus. It was found that using donors with erythroblastosis increases the mortality and reduces the latent period. On the other hand, continued passage of visceral lymphomatosis kept the incidence at a level not far removed from that of the first passage with visceral lymphomatosis the predominant lesion.

Material from birds with osteopetrosis was not used for transmissions because it was found that such material induced high osteopetrosis incidences but relatively little visceral lymphomatosis or erythroblastosis.

In a strain which had been passaged in the form of visceral lymphomatosis three times because of the lack of any erythroblastosis response, the fourth passage was made with material from the one case of erythroblastosis which occurred. This proved to be a very potent source of virus with a marked tendency to induce tumors of the reticulo-endothelial system. When extracts of the erythroblatosis-endothelioma material were used in further passages of this strain, this tendency remained a constant feature.

Inoculation of the original field material from two flocks were made in chickens obtained from three sources outside the Regional Poultry Research Laboratory. It was evident that none of the extracts induced a neoplastic mortality significantly above that in uninoculated controls. In L15I, however, there was a high incidence induced by both of these inocula. Inoculation of the same material in embryos of the same outside strain crosses is discussed elsewhere.

Virus from eleven sources was passaged in L15I chickens several times in order to try to characterize their oncogenic spectra and potency. In most of the strains both visceral lymphomatosis and erythroblastosis donor material was transmitted for a total of four passages. Third or fourth passage erythroblastosis material was titered for five of the eleven strains. Undiluted extracts of erythroblastosis liver induced about the same result in the titration as in the preceding passage, i.e., the third in which undiluted extracts were also inoculated. The result of the original passage of field virus is about equal to a -4 dilution of the titered fourth passage erythroblastosis material. In the other titrations similar results were obtained.

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Discussion

The results show that tumors occurring in commercial chickens raised under field conditions were good sources of oncogenic viruses. Material collected from about half of the source flocks had fairly high oncogenic potency in L15I birds. Although the material from the other sources was somewhat lower in oncogenic potency, there was only one inoculum which induced zero percent neoplastic mortality in inoculated chickens, the same zero incidence as in the uninoculated controls. There are two explanations for this variable response. The first is that there were insufficient amounts of virus in the low response materials or, secondly, that there were high titers of neutralizing antibodies in these tumor extracts. In vitro titrations of the tumor extracts indicate that those tumors giving a high initial response had high RIF activity. Low response extracts had low RIF activity but this may have been due to high antibody titers rather than to a lack of virus. The latter probability is now being explored.

Most of the materials came from flocks in which there had been high incidences of visceral lymphomatosis but it should be noted that mortality in the three flocks in California and the Albion flock in Michigan, which gave very low transmission rates, had relatively lower incidences of the disease. Not enough data is available, however, to allow one to make the statement that flocks with low incidences of visceral lymphomatosis have low content of virus in extracts of tumors from chickens in such flocks.

There was no correlation between the histological malignancy of individual tumors and the amount of oncogenic virus they contained, and tumors made up of lymphocytes with evidence of fibrosis in the infiltrated organ were fully as potent sources of virus as those which appeared much more malignant.

The lack of transmissibility of neural and ocular lymphomatosis can be interpreted in several ways, but our data indicates that material obtained from chickens with these diseases resembled a low virus yielding visceral lymphomatosis tumor. It is possible that with different handling, such as intraneural inoculation or embryo inoculation, a greater incidence of neoplasms and/or of ocular or neural lymphomatosis could have been induced. The result with the nephroblastoma tumor extract is not surprising in the light of recent work with the strain A, strain R, and the Murray-Begg endothelioma viruses which have shown that nephroblastoma is related to tumors of the avian leukosis complex from the etiological standpoint.

The transmission studies and titrations of passaged virus show that the initial extracts contained virus titers which could be increased by as much as 10,000 times after three passages as erythroblastosis extracts. When virus was transmitted in the form of erythroblastosis extracts there was also a widening of the oncogenic spectrum. Despite

this increase in virus titer, in no case was it found possible to induce visceral lymphomatosis in L15I chickens with a shorter latent period that had been possible using RPL 12 virus. Thus, while material was obtained from flocks in which there had been high visceral lymphomatosis incidences ranging up to thirty percent prior to twenty weeks of age, similar results could not be obtained using artificial means of infection in L15I birds.

The lack of success in inducing visceral lymphomatosis in commercial strains of chickens when they were inoculated as day-old chicks may explain why so many of the earlier workers failed in their attempts to transmit the disease.

Work with the avian tumor viruses from field sources has shown that they closely resemble the RPL 12 strain. This includes: the effect of route of inoculation; dose of virus; age of the host on the neoplasm incidence; increase in potency with continued passage of erythroblastosis extracts; and the reappearance of visceral lymphomatosis when these extracts were titrated and higher dilutions of virus were inoculated. In addition, the strains of virus obtained from field sources induced very few cases of neural lymphomatosis and none of ocular lymphomatosis which inoculated into L15I chickens. It appears that RPL 12 is generally as potent as any of the strains obtained from the field and that work heretofore done with RPL 12 applies directly to the virus strains causing visceral lymphomatosis in commercial operations.

Summary

Material from 22 flocks in various parts of the U. S. A. was collected for transmission studies. Tumor tissue from birds with visceral lymphomatosis was ground in a saline solution. Supernates from this homogenate served as the chief inoculum although in some cases high speed centrifuge pellets were also prepared from the supernates. In addition, oral washings were also prepared from birds in two flocks with high incidences of visceral lymphomatosis and used as inoculums. Material from nine sources induced more than a fifty percent neoplasm mortality in L15I chickens, and further passages were made to isolate and characterize these virus strains. Two other virus isolates with somewhat lower initial transmission results were also carried for further passages. Tumor extracts from eight flocks which induced less than a fifty percent neoplasm mortality response in the observation period of 245 days were not carried past the initial passage. Material obtained from chickens with neural lymphomatosis induced similar low incidences of visceral lymphomatosis as did those from chickens in a flock with a high incidence of ocular lymphomatosis. Extracts of a nephroblastoma had a high oncogenic potency inducing mainly visceral lymphomatosis.

Passage of material from the nine potent sources showed that by the selection of erythroblastosis donors, the oncogenic potency could be greatly increased. All shared the same oncogenic spectrum although there was considerable quantitative variation among the strain responses. Titration experiments and those involving the use of different routes of inoculation at different ages showed that the expression of the oncogenic spectrum was dependent on the dose, route of inoculation and age of inoculation as well as on the particular strain of virus.

The results obtained from this study show that the RPL 12 virus in all respects studied is similar to all other strains that have recently been isolated from field cases of visceral lymphomatosis.

The Oncogenic Spectrum of the Fowl Tumor Virus Strains and Some Influencing Factors

B. R. Burmester

Natural sources of visceral lymphomatosis virus, when inoculated by any of the more efficient routes into line 151 chickens, invariably cause largely visceral lymphomatosis, with a very low percent of erythroblastosis. By natural sources, I mean infection which is probably the source of virus in the natural spread of the disease, such as infected embryos, saliva, droppings, and incubator dust.

This compares favorably with the usual mortality picture that is seen under conditions of natural methods of exposure.

However, when more concentrated sources of virus, such as serum or tumor extracts are inoculated, one obtains a higher proportion of erythroblastosis and the appearance of other tumors and of osteopetrosis. This is further accentuated by stepping up the virus titer by rapid passage in susceptible chickens. This is well illustrated by the results presented by Dr. Fredrickson.

It has been found that several different factors have a marked effect on the relative occurrence of these neoplasms. It is of interest to examine these variations in order to understand more fully the biology of the avian tumor viruses, especially with respect to how these variations bear on the question of whether one virus causes the various neoplasms, or each is caused by a specific virus.

I believe you will agree that most of the data are much more easily interpreted on the basis of one virus causing several neoplasms.

Dr. Fredrickson has shown you some of the transmission results obtained from different parts of the country. There was found a considerable variation between different isolates and between strains in the number of different neoplasms observed after inoculation. On the other hand, there has been considerable uniformity. We have not observed any birds with myeloblastosis or with myelocytomatosis in any of our groups inoculated with field collected materials or in passages with such isolates. Neural and ocular lymphomatosis have been conspicuous by their absence. We have obtained occasional cases of neural lymphomatosis but at no time has a significant incidence occurred in any of the inoculated groups. This is despite the fact that several inoculums used have come from birds with neural or ocular lesions and some inoculations have been made into the embryo.

You have heard something about our new strains developed from field cases of lymphomatosis and no doubt you have heard something about strain RPL 12. This viral strain originated in a transplantable lymphoid tumor which was developed by Dr. Carl Olson, Jr., now of Wisconsin. During the first 6 passages only visceral lymphomatosis and osteopetrosis appeared in the inoculated birds. Erythroblastosis appeared in the 7th and increased as erythroblastosis donors were used for passage. Hemorrhagic endotheliomas appeared in the 14th passage and this, plus the erythroblastosis, VL, and osteopetrosis occurred in a series of passages.

Hemangioendotheliomas were eliminated by the use of donors without this neoplasm. Similar attempts were made to eliminate osteopetrosis and erythroblastosis but all such attempts have failed. The proportion of each type that occurs under standard conditions of dose, and route can be markedly altered by judicious testing and selection of donors; however, none of these has been eliminated.

Influence of Virus Dose

The dose of the virus inoculated has a profound effect on the proportion of the various neoplasms a particular strain of virus will induce. Results after inoculation with serial 10-fold dilution of RPL 12 virus shows that high doses cause predominately erythroblastosis which causes death within 100 days. Lower doses cause some E and some VL; still lower doses cause only VL. With these two neoplasms there is a clear separation related to time of mortality and dose of virus used. Very few VL cases die in less than 120 days after inoculation and almost none in less than 100 days. Similarly very few cases of E occur after 100 days of age.

Strain R virus has been studied for many years by many investigators in this and other countries. All have reported that this strain causes only erythroblastosis. However, when the dose of virus was reduced and the chickens held for longer periods, a different picture is presented.

The results of the low dose-long holding period experiment show that as the dose is reduced, so that there are some birds that survive the early occurring neoplasm, a high proportion of such early survivors later die of visceral lymphomatosis. Virus of strain R is also shed in the saliva and droppings causing E and/or VL in most of the chickens inoculated.

A similar situation was found with OAI strain A. This strain for many years was thought to cause only myeloblastosis, which it does when high doses are used and the birds are held only for the duration of the acute leukemic disease. Chickens that survive the early disease develop visceral lymphomatosis, renal carcinomas or nephroblastomas, as it is now called, and osteopetrosis. Oral washings and droppings extracts were again found infectious, causing all four types of neoplasms.

A fourth well known strain of virus, the Rous sarcoma virus obtained directly from the National Cancer Institute, was tested in the same manner. Again, this virus has been extensively investigated since 1911 when Dr. Peyton Rous discovered it. Again, when low doses of virus are used and the chickens held a long time, a significant percent develop visceral lymphomatosis. A few also died of erythroblastosis.

Thus the four strains of virus tested are obviously different in many ways, yet each causes visceral lymphomatosis, and three of the four cause a second common neoplasm, and that is erythroblastosis.

The simplest explanation for these results is that all strains are contaminated with a visceral lymphomatosis virus. This is quite logical because we and others have had sad experiences of virus strains getting contaminated and are actually "taken over" by the contaminating virus. Also, we know that the virus of VL occurs widely and most often as an inapparent silent infection. However, such an explanation does not seem to fit all the facts, though this is not denying that some so-called strains or isolates contain more than one virus. Another argument against the contamination explanation is that strains R, A, and Rous sarcoma have long been passaged rapidly allowing little or no buildup of a lymphomatosis virus, which is known to multiply relatively slowly.

A marked influence of virus dose can also be demonstrated with the more pleomorphic strains recently isolated. Results with strains RPL 26, 28 and 29 at a high and low dose are of particular interest. All three strains confirm the reciprocal occurrence of E and VL so clearly shown with strain RPL 12. When the dose is high, most of the chickens die of erythroblastosis; when the dose is low, a high percent of the survivors die of VL. At the high doses, in addition to erythroblastosis, a variable proportion develop fibrosarcomas, hemangioendotheliomas, and many also have extensive hemorrhages. These tumors largely disappear with decrease of virus dose before the disappearance of erythroblastosis and VL.

Influence of Age at Inoculation

The age of the chicken at the time of infection is another factor which markedly influences the response of chickens to the tumor viruses. There is a gradual and continuous decrease in the susceptibility of chickens to infection as they grow older. The dose of virus to cause VL in 50 percent of the chickens inoculated intravenously increases from a low of -9 logs at 1 day to a -6 logs at 84 days. When the disease response is measured in terms of percent erythroblastosis, a parallel change is observed except that during the first 2 weeks there was no significant change. Then the dose required for an LD_{50} increased by about 2 1/2 logs.

When the virus is given by the intraperitoneal route there is a much more rapid increase in resistance or loss of sensitivity. By 3 weeks of age the chicken is about 1000 x more resistant by this route than at 1 day. It should be mentioned that the so-called intraperitoneal route in the chicken is a misnomer. Injections made into the abdomen of the chicken are deposited primarily in one of the abdominal air sacs, which of course is a part of the respiratory system of the chicken.

Chickens infected by the oral and nasal route, in comparison with the intravenous route, become resistant much more rapidly with age. This shows that the increase in resistance to visceral lymphomatosis inducing viruses, which is related to aging, varies with the route of inoculation or exposure, and that such changes are more obvious with the natural routes of exposure.

Age at inoculation also has a marked effect on the tumor spectrum. The occurrence of osteopetrosis is highly conditioned by early age of infection. When strain RPL 12 virus preparation L29, which has a high osteopetrosis potential, is inoculated into chicks at 1 day of age, 98 percent developed osteopetrosis. Inoculation of the same preparation at 7 and 21 days resulted in much less--22 and 4 percent respectively. The proportion with erythroblastosis in this experiment actually increased with age. This was an indirect effect due to the marked decrease in chickens affected with osteopetrosis. There appears to be a definite interference between these two diseases and it would seem to be at the lesion level rather than virus.

In another experiment virus of strain RPL 12, preparation L31, when injected intravenously into embryos, caused, in addition to a high incidence of erythroblastosis, other kinds of tumors such as sarcomas, hemangioendotheliomas, and nephroblastomas. When inoculation is delayed to one day of age, only erythroblastosis plus a little osteopetrosis developed. Further delay to 14 days of age, with constant dose and route, resulted in decrease in erythroblastosis, absence of osteopetrosis, and occurrence of VL at moderate levels.

Results with other strains of virus such as RPL 26 and 29 demonstrated that embryo inoculation should be included in any assessment of the oncogenic spectrum of the avian tumor viruses. Some strains such as RPL 26 will produce many different tumors when chickens are inoculated, but other strains such as RPL 12 and 29 require a more sensitive host such as the embryo for the induction of the solid tumors. Here is a good example of some of the differences between similar strains of virus. This difference is not due to potency or virus concentration. Two strains may be the same as measured by one criteria such as induction of erythroblastosis, but there may be a wide difference in their ability to induce other tumors such as between strain 12 and 26.

Influence of age at inoculation is even more marked in chickens other than line 151. One experimental stock of White Leghorns--A--and two commercial strain crossbreds--B and C--were inoculated as 11-day old embryos and as day-old chicks. In one experiment undiluted strain RPL 12 virus inoculated into embryos caused up to 85 and 90 percent of the chickens to develop various types of neoplasms, but mostly erythroblastosis. In contrast, only one-third of the day-old chicks of the same source developed tumors. The number of different kinds of tumors was also less. This difference was further accentuated when a 1:100 dilution of virus was used. Ninety-four percent of the embryos inoculated developed tumors but only 11 percent of the chicks inoculated at 1 day, later grew tumors.

Similar data were obtained with field virus, i.e. unpassaged virus. Akron (Circleville) virus caused more neoplasms in stock A and C chickens when inoculated as embryos than when inoculated into chicks. Similar results were obtained with Strickland virus.

In all groups the erythroblastosis caused death in a shorter interval when inoculations were made into embryos; however, visceral lymphomatosis did not appear any sooner when inoculations were made into embryos in comparison with day-old chicks.

Influence of Route of Inoculation

Route of inoculation has already been mentioned as one of the factors which affect the proportion of various tumors induced.

An example of this is found in the results of an experiment involving the inoculation of line 151 chickens with strain RPL 26 by the intravenous, intraperitoneal, and intramuscular routes. Chickens 1 day and 14 days of age and a -2 logs and -4 logs doses were used. Within any one dose-age group, there was a consistent marked increase in sarcomas when the route of inoculation shifted from

intravenous to intraperitoneal to intramuscular. There is some suggestion of a reciprocal tendency in the occurrence of other tumors, and for the occurrence of hemorrhages. This is especially indicated when erythroblastosis is at critical levels.

When the circumstances are such that the neoplasms are primarily erythroblastosis and/or visceral lymphomatosis, the effect of route is primarily one of efficiency of transfer of virus to the sensitive cells. Chickens inoculated with a large dose of RPL 12 virus developed 93 percent erythroblastosis and no visceral lymphomatosis. An equal number of non-inoculated chickens reared in direct contact with the inoculated chickens developed VL in 51 percent of them, with none showing evidence of erythroblastosis thus indicating that the virus given by an experimental method causes all erythroblastosis but when transmitted by natural means causes only visceral lymphomatosis--the usual natural occurring disease.

Discussion

Studies of tumor and other infectious material from 22 different flocks and 11 new strains have shown that the viruses isolated are all similar in their oncogenic potential and they are also similar to the older strains RPL 12, RPL 16 to 23 and strain R. They all cause erythroblastosis and visceral lymphomatosis. In addition, they all have caused other similar tumors.

These observations in themselves are strong circumstantial evidence that there is essentially a single virus which was isolated in each instance and the virus in each isolate was essentially the same. It then follows that a single virus is responsible for many different types of neoplasms.

Undoubtedly some of the effect of route of inoculation can be accounted for by the more or less efficient transfer of the virus to susceptible cells. Thus, strain RPL 26 which has a marked fibrosarcoma potential when administered by the intramuscular route, causes a very high percent of the chickens to develop fibrosarcomas at the point of inoculation, whereas when given by the intravenous route, the incidence is much lower.

Such an effect is much less marked or hardly discernible when virus strains having a low fibrosarcoma potential were employed in a similar experiment.

The consistent shift in the types of neoplasms obtained with a decrease or increase in the amount of virus inoculated also suggests a single entity. Similarly, a shift in the oncogenic spectrum, with a change in the age at inoculation, is most easily explained on the basis of a change in cell sensitivities of the host.

Dr. Beard has studied BAI strain A virus extensively for several years and has detected no evidence for the presence of more than one virus even though this strain causes 4 distinct neoplasms and Dr. Baluda has provided strong evidence that these 4 neoplasms are caused by the same virus.

Although the overall data definitely point to a single virus that can cause erythroblastosis, visceral lymphomatosis, osteopetrosis, fibrosarcomas, hemangioendotheliomas, and nephromas; there is also definite indication that there are minor yet notable and continuous differences in the pathologic characteristics between viral strains that have arisen from chickens with the same disease. It is quite probable that such differences may not reflect differences in antigenic constitution and thus would be of only minor importance. However, these differences are quite stable provided the right donors are used for propagation of the strain, indicating that they have a genetic basis. The "right" donors are not discernible by gross or microscopic examination and proof of the potential of any inoculum is always dependent on an in vivo test, i.e. the inoculation of susceptible chicks and/or embryos with serial 10-fold dilutions of virus and holding the chickens for an extended period.

Neural lymphomatosis, ocular lymphomatosis, and myelo- or granuloblastosis, as well as myelocytomatosis, have been conspicuous by their absence. The techniques and recipients which have been successfully used in transmitting and markedly increasing the occurrence of all other virus neoplasms of the chickens have not been successful in causing the neural or ocular lymphomatosis and neoplasia of the myeloid elements. This has been the result despite the facts that (1) the recipient chickens are known to be susceptible to development of these diseases, and (2) various materials from chickens with ocular or neural lymphomatosis have been used. Even through these negative results have been repetitive and overwhelming, they cannot be taken as proof of separate etiology.

Pathological Findings in Mammals Inoculated with Avian Visceral Lymphomatosis Tumor Suspensions

Olive Stull Davis[1]/

Pathological changes were found in sheep, guinea pigs, and mice inoculated within a few days after birth with suspensions of tumors from chicks infected with virulent strains of the virus of avian visceral lymphomatosis. Similar changes were not found in control animals.

In all experiments inocula consisted of 1:10 suspensions in sterile saline solution of breast tumor and tumorous liver from chicks in serial passages of AVL of Regional Poultry Research Laboratory strains 12 or 16. With two exceptions when groups of guinea pigs and mice were inoculated once only, animals were inoculated i. p. twice weekly for a total of 10-21 times in the various experiments. Controls were similarly inoculated with comparable normal chicken tissues, or with sterile saline solution, or were uninoculated.

Tumor-inoculated and control animals selected at random were sacrificed and necropsied at intervals of from 1 1/2-13 months in mice, from 1-20 months in guinea pigs, and from 8-38 months in sheep. A wide variety of tissues were examined histologically, including many regional lymph nodes.

The results in all three species studied were remarkably similar. In mice and guinea pigs which were inoculated only once, results were similar to those in animals inoculated 10-21 times. Some histological changes were found in all AVL-inoculated sheep, in all AVL-inoculated mice kept at 2 1/2 months or longer, and in all AVL-inoculated guinea pigs kept three months or longer, and in some animals kept for shorter periods. These changes varied from slight to very marked and extensive changes in several different organs, sometimes correlated with gross enlargement of the organs involved, such as lymph nodes, spleen, and liver. In general, the changes were greatest in animals kept for the longest time after the initial inoculation with avian tumor material.

The following kinds of histological changes were found most frequently:

1. Lymphoid and reticulum cell hyperplasia was seen in various lymph nodes, often with loss of follicular structure, and frequently with masses of lymphoblasts, lymphocytes, and

1/ Department of Veterinary Anatomy, School of Veterinary Science and Medicine, Purdue University, Lafayette, Indiana

reticulum cells, and in a few cases many plasma cells, filling the medullary sinuses. This picture was similar to the Potter lesion described in biopsy studies of lymph nodes of preleukemic mice by Potter and co-workers. In some lymph nodes, lymphoid cells were found in the peripheral lymph sinus, in the capsule, and in the surrounding connective tissue outside the node.

2. Varying degrees of lymphoid hyperplasia were seen in other lymphoid organs, such as spleen, thymus, and lymphoid nodules of the stomach and intestinal walls.

3. Slight to extensive lymphoid infiltration was found frequently in the kidneys, lungs, liver, and salivary glands, and less often in the heart, trachea, thyroid gland, adrenal, ovary, oviduct, and pancreas.

4. Many cystic tubules in the kidneys were seen, sometimes with marked hypertrophy and proliferation of the epithelial cells.

5. Hepatoma-like hypertrophy and proliferation of liver parenchymal cells often appeared, particularly in the periphery of the organ and around the central veins, with ballooning, vaculation, and distortion of the epithelial cells, and often with stratification of smaller flattened cells in the liver cords, often between masses of the large epithelial cells.

6. Hyperplasia of the parenchyma of the salivary glands, particularly the parotid, with loss of architecture, was sometimes seen.

In some groups of chicks inoculated with suspensions of viscera from AVL-inoculated mammals, in other experiments not reported here, the incidence of AVL was significantly higher than in comparable control groups of chicks, suggesting that the AVL virus survived in mammals for several months, even when no gross lesions were visible.

IN SUMMARY:

The pathological findings in all three species studied showed considerable consistency. In most cases, they were greatest in animals necropsied longest after the initial inoculation with avian tumor material, so that the pathological process appeared to be progressive. Similar changes were not found in control animals kept for the same periods. For these reasons, the changes found appear to me to be significant.

Leukosis Research at Penn State University

R. F. Gentry[1]/

Field isolates of visceral lymphomatosis virus have been used in an attempt to infect various strains of chickens. During 1960, filtrates from 10 field outbreaks were prepared and inoculated into 4 commercial crosses (experimental) that had shown a high natural susceptibility to leukosis and the RPL line 15I susceptible birds. The RPL 12 strain of virus was used as a positive control. The results shown in Table I were all negative for transmission except in the highest concentration of the RPL strain 12 in the RPL line 15I birds.

TABLE I

Source of Birds

Inoculum	X-1	X-2	X-3	X-4	Line 15I	Total
21	2	3	0	4	5	14
22	2	1	0	4	1	8
St.12 10^{-4}	2	2	4	4	17	29
St.12 10^{-6}	1	4	3	7	7	22
23	3	0	1	3	7	14
24	1	0	1	3	4	9
25	2	2	2	2	1	9
40	3	1	0	7	9	20
28	0	1	1	3	1	6
42	1	1	1	1	1	5
43	0	0	2	2	0	4
44	1	3	1	2	3	10
Isol.cont.	1	0	1	3	1	6
Total	19	18	17	45	57	136

13 inoc. X 5 sources X 35 birds/group = 2275 total birds.

It was concluded that the concentration of virus in the field isolates was not sufficiently high to produce infection in the strains of birds employed.

In order to improve our chances of success, the work for 1961 was designed to employ field isolates that had first been screened at the Regional Laboratory and shown to be infective to the RPL line 15I birds. Three of these isolates and RPL strain 12 virus were inoculated into selected strains of birds. Using the incidence of early infection in the 1961 Pa. Random Sample test as a measure, eggs from

1 Pennsylvania State University, University Park, Pennsylvania

4 commercial strains of birds were purchased. Also the neural leukos.s susceptible strain from Cornell and RPL line 15I were used. As shown in Table II all groups had a high incidence of neural infection.

TABLE II

	Source of Birds					
Inoculum	Cornell	DeKalb	Good	Garrison	Kimber	Line 15I
Akron	24/4*	8/11	4/16	3/7	6/9	13/27
Strickland	26/4	3/8	5/17	5/8	-	6/28
Pierceton	-	3/7	-	2/13	-	5/28
RPL 12	17/9	7/14	9/17	4/10	6/14	7/26
Contact Control	-	-	-	5/4	-	10/11
Isolated Control	19/3	4/10	9/1	5/8	6/8	11/17

* LN/LV
Line 15I - 50 birds/group, all others 35 birds/group

In many groups the early mortality with neural was so great there were not sufficient birds remaining to allow a measure of visceral infection. The general spread of infection in all groups is suspicioned to be a field type infection independent of the materials inoculated. The high incidence in the Line 15I isolated control group prevents an accurate evaluation of the response in the inoculated groups. This is more confusing when it is realized that the same birds were inoculated with the identical virus isolates at the Regional Laboratory and a very low incidence of infection occurred in all groups with practically no neural infection.

Due to the relatively high cost of this type of work, our project for 1962 has been designed along different lines. Routine use of the Fluorescent Antibody technique in our laboratory makes it more logical for us to pursue this type of approach. Embryo inoculation of virus will be used to screen for high titered antiserum which will then be utilized for Fluorescent Antibody tests.

Avian Lymphomatosis I. Experimental Reproduction of the Neural and Visceral Forms

Martin Sevoian[1]/

Abstract[2]/

Combined neural and visceral lesions of avian lymphomatosis have been consistently reproduced in 90 to 100% of S strain White Leghorn chickens with isolate JM within 2 to 3 weeks after inoculation. Evidence is presented that these manifestations may be caused by the same agent. These findings give emphasis to the significance and prospective use of the chick as an experimental animal in cancer research.

1 University of Massachusetts, Amherst, Massachusetts

2 This abstract is of a paper prepared by Dr. Sevoian, D. M. Chamberlain and Fred T. Counter which will be published soon. Dr. Sevoian discussed informally the findings which will be presented in the paper.

Recent Studies on Rous Sarcoma Virus

Howard M. Temin[1]/

Chickens infected with Rous sarcoma virus (RSV) develop tumors. Presumably, the cells of the chicken infected *in vivo* with RSV become tumor cells. Chick cells infected *in vitro* with RSV become converted in morphology and in growth properties. The change in morphology is most easily seen in infection of cells of the iris epithelium of the chick embryo. Normally these cells grow as a monolayer of closely opposed epithelial cells, usually containing pigment. If such cells are infected when growth is possible, they change to long, fusiform cells or round, refractile cells depending upon the strain of RSV used. The cell can have some influence upon the morphology of the infected cell as is seen especially when chick cells and duck cells infected by the same virus are compared. The virus also changes the growth properties of the cells. Normally, if a small number of chick cells are plated upon a feeder layer of irradiated mouse cells only about 10% will grow into clones. However, if after plating these cells are infected with either strain of RSV the cloning efficiency goes to 50-100%. The clones are of converted cells and their number varies as the dilution of virus used for infection. A change in growth properties also occurs when a whole culture of chick cells is infected.

From experiments like these we can draw a picture of virus-cell complex in RSV chick fibroblast infection. A single virus infecting a cell becomes integrated with the cell so as to change the morphology and growth properties of the cell. The cell transmits the virus genome regularly to its daughter cells and all cells release virus and divide. Such a picture assumes both integrated virus and autonomously replicating virus. Recent work has shown that cells carrying only integrated virus can be produced, suggesting a control mechanism reminiscent of that in bacteriol lysogeny. If chick or turkey cells are infected with less than 1 FFU per cell of RSV of either strain and are grown in the absence of large numbers of uninfected chick or turkey cells, in about half of the experiments the foci of converted cells resulting will not be virus producing. The requirement for absence of chick cells is necessary only for an initial labile period of a few days. The requirement for only 1 FFU of RSV is absolute.

1 McArdle Laboratory, University of Wisconsin, Madison, Wisconsin

The converted, non-virus producing cells (CNVP) are not releasing an interfering virus or viral neutralizing substances, do not contain intracellular virus, virus particles or large amounts of viral antigen. They do transmit the specific conversion to all their progeny cells and do contain information to make virus. Mature virus appears spontaneously with a low frequency per cell generation or in most cells after treatment with other strains of Rous virus, erythroblastosis virus and avian myeloblastosis virus. The inducing ability of Rous virus is killed by agents at the same rate as focus-forming ability and behaves the same in centrifugation. The inducing ability can be titrated by the amount of RSV produced, giving a nearly linear relationship. With large amounts of inducing virus all cells release the original virus when plated as infective centers.

These results suggest a comparison with lysogeny and bacterial episomes. In many respects there are similarities and so the names virogeny and paraepisome may be used.

It then may be asked what property of the paraepisome is responsible for carcinogenesis. When CNVP are injected into the chorio-allantoic membrane of chick embryos or into the head of day old chickens, fibro-sarcomas appear. In five chorio-allantoic and three chick tumors there was no virus present. These results indicate that viral production is irrelevant to carcinogenesis and that integration or conversion, if these are separable, is responsible.

	Med	r/1	r/4	r/16	r/64	
Rous	0	167	105	48	12	
Fib	10	13	22	14	12	
	Med	f/1		f/16	f/64	f/256
Clones	8	131		134	94	70

About 200 chick cells plated on 4×10^5 irradiated mouse cells were infected with various dilutions of RSV. The resultant clones were counted. The values are the mean of two plates.

λ	F	RSV
extra-, intra-cellular	intra-cellular	extra-, intra-cellular
DNA		RNA
Two states	Three states	Two states
productive (cell death, not integrated)	productive (cell division, not integrated)	
	productive, integrated	productive (cell division, integrated)
non-productive, integrated	non-productive, integrated	non-productive, integrated
Conversion	Conversion	Conversion
immunity	?	immunity
induction	induction	induction
spontaneous	spontaneous	spontaneous
by irradiation, stoppage of DNA synthesis	?	not by irradiation, stoppage of DNA synthesis
not by superinfection	?	by superinfection
High moi leads to non-productive	?	High moi leads to productive
Chromosomal localization	Chromosomal localization	? localization
Lysogeny-episome	Episome	Virogeny paraepisome

Studies on the Natural Modes of Transmission at the Regional Poultry Research Laboratory

B. R. Burmester

Studies at the Regional Poultry Research Laboratory on the natural modes of transmission have been almost entirely confined to visceral lymphomatosis. This was not by design but because the natural incidence of neural lymphomatosis at the RPRL has been quite low and we have not obtained a significant incidence of the disease upon inoculation.

Our data show that there are two important ways in which the virus is transmitted, i.e. through the egg and by bird to bird contact.

Our first direct evidence that virus was in the egg was in experiments designed by Dr. Cottral while he was at this Laboratory.

Eggs from individual hens were marked, incubated to 14 days of age, the liver removed, an extract prepared and injected into groups of susceptible line 151 chicks to test the induction of disease. In titration experiments it has been shown that the amount of visceral lymphomatosis induced is proportional to the amount of virus inoculated.

The induction of VL by the different extracts varied from a low of 12 percent to 56 percent.

The high incidence of VL obtained with eggs of some of the hens definitely pointed to virus in the embryos.

A year later similar experiments were conducted with chickens selected at random from 3 inbred lines. The height of the columns indicate the percent visceral lymphomatosis induced in chickens with embryo extracts of individual hens.

Only one of 4 hens of resistant line 6 was found to be a shedder of virus whereas all of 9 hens of line 9 and 7 of 8 hens of line 15 both of which were classified as susceptible lines, were considered shedders on the basis of infected embryos. Chickens injected with embryo extract from some of the hens developed up to 65 percent with visceral lymphomatosis.

Similar tests were made on the hens that were laying at 15, 18 and 25 months. All except one of the hens tested at 15 and 18 months of age were found to be heavy shedders. When tested at 25 months, all were either light shedders or non-shedders.

Progeny of some of the shedder hens were tested and most were found to be heavy shedders when tested at 8 months but not at 12 months.

It thus would appear that hens shed virus into their eggs so the embryo becomes highly infected. Most hens of infected and genetically susceptible stock were heavy shedders. This continues over long periods though it does not appear to be a lifetime infection. Termination of infection or, at least, shedding of virus is somewhat variable and the factors causing its termination are not known.

Occurrence of lymphomatosis in relation to shedding of virus is of interest. The hens tested were divided into those that were heavy shedders, light shedders and non-shedders. During one year following the first test, 3 of the heavy shedders died of VL, but none of an equal number of light shedders developed tumors.

Six to twelve sisters of hens tested were reared in the same pens. Those of the heavy shedders had an incidence of 36 percent, light shedders - 13 percent and non-shedders 0 percent lymphomatosis,

Five sets of progenies of the tested dams were obtained. One was reared in the regular genetics population, the second was reared by family isolation. The latter population as a whole had a very low incidence. However, in both populations the progeny of the heavy shedder dams had a higher incidence of visceral lymphomatosis than those of the light shedders or non-shedders. There were, however, several families of heavy shedder dams that had no cases of VL and there were a few families with one or two cases of VL that came from dams classified as non-shedders.

The significance of egg transmission becomes much more evident when we realize that it is the source of infection that spreads by contact in the hatching and brooding units.

An experiment conducted by Dr. Waters shows very well the importance of contact transmission under natural condition of infection. Chickens of line 151 stock were exposed in the incubator, in the brooder unit at 1 day, at 10 days, at 20 days and at 30 days of age and thereon. The stock reared in isolation developed only 10 percent VL, those exposed in the incubator and thereon had 77 percent VL and the rate was less for other ages being only 15 percent when exposure was delayed until 30 days of age.

Further evidence for presence of infection in the hatching unit was obtained in another experiment wherein chickens of a non-infected stock when inoculated with incubator debris extract developed VL to the extent of 57 percent. The controls only had 2 percent and surprisingly the chickens that hatched during the collection of the debris were of lines 9 and 15 and tumors developed in less than 7 percent.

Infectivity tests were made of oral and fecal extracts. They showed that oral washings from chickens with visceral lymphomatosis were highly infectious, likewise fecal extracts were also infectious.

Similar tests showed that oral washings and the droppings of some normal appearing chickens were also infectious, thus demonstrating that chickens shed virus in their normal body secretions and excretions.

In an experiment in which chickens were inoculated and others placed in contact, it was found that virus at high levels was found at the first sampling of saliva and that was 10 days after infection. Chickens in contact took much longer. Not until 90 days after contact with inoculated chickens was there significant shedding of virus. Even at this time it was over 100 days before any of the contact chickens developed lymphoid tumors.

Experiments with a virulent strain of virus showed that infection ending in tumors is obtained when virus is applied to any of the mucous membranes of the natural body openings of the bird. These include the mouth, eye, nose and cloaca. When virus was sprayed into the air of an enclosure containing the chicks to be exposed, leukosis resulted. Visceral lymphomatosis developed in 40 to 70 percent of chickens exposed by these natural routes. Virus placed directly in the esophagus did not result in a significant incidence. This may be related to the high acidity of the crop contents.

Since the virus covering visceral lymphomatosis spreads from bird to bird within the same population, and since experimentally airborne virus can infect and cause tumors, the virus must be considered contagious in the usual sense. However, controlled experiments show that it is not nearly as contagious as the more usual viruses such as Infectious Bronchitis, Laryngotracheitis, Newcastle Disease, etc.

Thus data of one experiment shows that the exposure dose, i.e. the proportion of infected birds in a given population at a given age has a marked influence on the amount of transmission to pen mates that occurs. Another factor of marked importance is the age of exposure. The younger the bird at the time of exposure, the greater the disease incidence. This effect is no doubt due to the more general phenomena that as chickens get older, physiologically they become more resistant to infection.

We have conducted numerous experiments in attempts to determine just how the virus is transferred from bird to bird in the same pen. Thus far we have not succeeded except to show repeatedly that infection as measured by tumor induction occurs only when there is a direct body contact, i.e. an intermingling of infected and exposed birds. No transfer of infection could be detected by these means when chickens: (a) drank out of the same water fountain, (b) ate out of two different sides of the same feed trough, or were placed each morning on overnight droppings of inoculated birds. These negative data imply that body contact is necessary for the horizontal spread of virus. This, however, is not a conclusion. Most of us have made observations in the field which suggest that horizontal spread of infection may take place without body contact.

Summary

Our data show that there is no question concerning the importance of egg transmission of visceral lymphomatosis and the contact horizontal spread of infection in the hatching and brooder units. Egg transmission is unusually important in this disease because hens shed virus into their eggs for long periods during which time there are no signs of disease. Such chickens are ideal means of maintaining the disease. Contact spread of infection becomes important in the incubator and brooder units when a high proportion of the chicks are genetically susceptible and at the same time are without a passive immunity due to antibodies transferred by the parent.

It is quite obvious that much more needs to be learned concerning the natural transmission of visceral lymphomatosis.

Epidemiology of Leukosis Virus in Commercial Operations

W. F. Hughes[1]/

Recent studies conducted by the University of California virus laboratory and Kimber Farms indicate that the virus associated with the avian leukosis complex behaves epidemiologically as other infectious poultry viruses.

It has, however, some properties which make it difficult to conceive of it as a classical disease virus. First and foremost is its lack of pathogenicity. Excluding the area of tumorogenesis, the virus is nearly, but not quite, non-pathogenic. Second, it apparently spreads at a slow rate. In an earlier discussion it was pointed out that competent chicks hatched and housed with tolerant chicks which were shedding at a high rate, showed the first detectable infection at seven weeks of age and the first antibody at nine weeks of age. The pullets were 18 weeks of age before a high proportion of the competent chicks were protected by antibody. Third, as a result of the low pathogenicity chickens may survive indefinitely while infected with the virus. The recently discovered state of immunological tolerance results when transovarian transmission of the virus occurs. The tolerant chicks are continuous sources of virus infection for chickens in contact.

In mature flocks of the Kimber experimental line, most of the competent hens maintained circulating antibody, and virus is undetectable in either body fluids or embryos. There is, however, a small number of hens which shed virus into occasional embryos in spite of reasonable levels of circulating antibody. These comprise about 10% of the Kimber leukosis susceptible population.

All studies of virus and antibody by the Rubin technique thus far reported have been made on experimental populations. There are several reasons why these results may not be typical of commercial chickens.

1. The chickens have been selected for high incidence of neoplasia. The basis for the susceptibility is not known.

2. Early heavy exposure may modify the chicken's ability to develop antibody to leukosis virus as has been described by Wolfe (1) for non-living antigens.

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1 Kimber Farms, Fremont, California

3. The rate of shedding is far higher in this population than for commercial operations and probably provides a different stimulus than that encountered in the commercial environment.

A study of the epidemiology of leukosis virus in breeding and commercial flocks should provide important clues on how to approach the problem of leukosis control. There are important problems of the relationship of the virus and tumor which remain unresolved; but information on the age at infection, rate of spread and development of immunity will contribute to the development of biological control.

Kenzy studied antibody to Rous through the RVN test and found antibody in survivors of leukosis affected flocks and he found some mature flocks without detectable antibody. In a subsequent paper (3) he described development of Rous neutralization antibody in birds brooded in contact with possible transmitters. Kenzy (4) has published two papers on the use of the RVNA test on adult populations. In both papers flocks with high leukosis losses tend to have high Rous virus neutralizing antibody and low loss flocks tend to have low levels of antibody to Rous.

Infection studies by the Regional Poultry Laboratory Group have been discussed elsewhere.

Materials and Methods

Tests for virus and antibody are by the technique of Rubin (5), which involves invitro interference with Rous virus for the detection of leukosis virus and a tissue culture neutralization test of Rous virus as an estimation of leukosis antibody.

Serum samples were obtained from 13 flocks varying in age between two weeks and 12 months. Nine of the samples were tested in the Kimber Farms laboratory and four at the University of California laboratory.

The samples reported here were obtained for other laboratory purposes and though we have initiated a survey to gather data on the age of infection and rate of spread of leukosis virus in breeding and commercial flocks we have been unable to apply coordinated effort to this project. As a consequence there are no repeat samples or orderly sequence of ages. The results are presented by the age of the chickens.

Results

Table 1 presents the accumulated data on virus or antibody tests on the 13 flocks.

In this small sample neither virus or antibody were detected in chicks 7, 9 and 11 weeks of age but virus was detected in a second sample 9 weeks of age.

All flocks 16 weeks of age and older showed evidence of contact with the virus. About half the individuals tested in three flocks between 9 and 11 months of age were without antibody. This level of protection is insufficient to prevent additional spread of virus in that flock.

Discussion

Studies on the Kimber Farms leukosis experimental flock suggests that the virus of leukosis is epidemiologically similar to other infectious viruses. Infection is followed by a period of viremia, antibody is developed which removes the virus from the circulation.

Investigations by Burmester suggest that spread between hosts requires close contact and thus spread within the flock may be quite slow compared with, for instance, infectious bronchitis virus. A slow spread of infection within the flock is also indicated by Rubin's results and is not contra-indicated by the present study.

The results indicate that the virus is quite wide-spread among adult flocks. Antibody was found in every lot of chickens over 16 weeks of age. There may be some conflict with Kenzy's data on this point; however, it is likely that there are sharp geographical differences in the incidence of virus infection.

The age at which the immunizing infection occurs is of great importance to poultry breeders. Rubin has established that viremic hens shed virus into nearly every egg; therefore, we can assume that hens which receive the immunizing infection after sexual maturity will shed the virus in her eggs during the period of immunizing viremia in much the same manner that avian encephalomyelitis virus is shed in eggs during the immunizing infection. There is insufficient information to assess the related risk of hens with antibody which shed the virus as has been found in our experimental population.

The data in Table 1 suggest that there is danger of infection occurring after sexual maturity in breeding hens. Three lots of hens between sexual maturity and 12 months of age did not have anywhere

near complete protection. It therefore appears that in breeding operations quarantine with the purpose of excluding the leukosis virus infection or some means of immunizing the pullets prior to sexual maturity will reduce the known risk of egg transmission of leukosis virus not the cause of acute outbreaks.

It appears likely on the basis of unpublished data that the presence of circulating virus is involved in the general stress of growing birds, probably the specific ability of the bird to defend herself against other infections. If this proves to be the case with further investigation it would provide one additional reason for controlling the exposure, or the timings of exposure, of chickens to leukosis virus.

Summary

Thirteen lots of serum from chickens between two weeks and 12 months of age were tested for leukosis virus and antibody or both. All lots from chickens 16 weeks of age and older showed evidence of infection with leukosis virus. Some implications of the findings are discussed.

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Table I

Flock	Ident. KF Acc. No.	Date	Age	No. Tested	Virus No.	Virus %	Ab. No.	Ab. %	Remarks
1	453	1/18/62	2 wk.	10	Not done		2	20	Passive antibody.
2	450	12/19/61	7 & 11 wk.	9	Not done		0	0	Paralysis 5/9 had swollen nerves.
3	464	2/15/62	9 wk.	6	0	0	Not done		All lame, isolates SP plant.
4	454	1/19/62	9 wk.	6	2	33	Not done		Increase in mortality; 3 with tumors.
5	457	2/20/62	16 wk.	21	0	0	3	15	Reactors to pullorum antigen (not infected); Raised in isolation. Not doing well.
6	Rubin 1960	1960	6 mo.	16	0	0	11	70	Brooder in isolation.
7	Rubin 1960	1960	6 mo.	18	0	0	17	94	Brooding crowded - in isolation.
8	415	8/8/61	27 wk.	7	Not done		6	85	Comml. High leukosis loss, unsatisfactory production.
9	Rubin 1960	2/60-12/60	Mature hens	550	15	3	Not done		Embryos of 1 strain, probably many ages of birds.
10	430	9/8/61	Mature	4	Not done		2	50	Different kinds of tumors.
11	457	1/24/62	10 mo.	12	Not done		7	60	Poor production. No obvious reason.
12	465	2/15/62	11 mo.	16	1	6	10	60	Same as above, different house.
13	Rubin 1960	1960	12 mo.	25	Not done		25	100	Normal flock.

Avian Leukosis Complex in Young Chickens at the Time of Processing 1/

William J. Benton 2/

The problem of Avian Leukosis as a cause of condemnations in broilers first came to our attention in 1956 when the Federal Poultry Inspection Service referred specimens with various lesions of leukosis including skin and muscle lesions to our laboratory. Studies at that time confirmed the presence of visceral lymphomatosis (Benton and Cover, Avian Dis. I, 3, 320-328, 1957).

Since that time a survey has been concluded concerning carcasses condemned due to leukosis by the poultry inspection service in two areas of the country in an effort to determine the incidence of the various forms of the disease and to study in detail the skin and muscle lesions. This survey included 92 randomly selected flocks totaling 784,429 broiler age chickens from the Delmarva area during a period from November 1959 through May 1961 and included specimens from four processing plants. In addition, a more limited survey including 32 flocks totaling 223,276 chickens from four processing plants in the north Georgia area was conducted over a 1 week period in April of 1961.

Randomly selected carcasses showing definite or suspicious skin or muscle lesions were critically examined and tissues for histopathologic studies were obtained from affected areas of skin, muscle, all other grossly affected organs and routinely from several of the viscera regardless of gross appearance.

The results of these studies have been compiled and show that the average condemnation rate due to all disease processes for the Delmarva area was 1.20% (range of 0.29% - 8.16% and median of 0.88%); for the north Georgia area, the same respective values were 1.69%, 0.17% - 14.4% and 0.92%.

On a flock basis in Delmarva, the various forms of the leukosis complex accounted for an average of 34% of the carcasses condemned because of disease with a range of 3.5% to 72.5%; in the north Georgia area, the respective values were 15% and 0 to 38.4%.

1/ This research was supported by a research grant (C-443(C2)) from the National Cancer Institute, National Institutes of Health, Public Health Service.

2/ University of Delaware, School of Agriculture, Newark, Delaware.

The incidence of various lesions of the disease in leukosis condemned carcasses is shown in the following tables where for the purpose of pointing out the specific involvement, all carcasses exhibiting skin or muscle lesions are placed in that category irregardless of visceral or osteopetrotic involvement.

Area		Percent Leukosis Condemned Carcasses with Gross Lesions in: Skin	Muscle	Viscera	Bone
Delmarva	Average[1]/	20.5	1.4	38.0	37.2
	Range	0 - 75	0 - 26	0 - 83	0 - 95
	Median	18	0.7	35	37.5
N. Georgia	Average	6.4	0.7	35.5	54.3
	Range	0 - 75	0 - 11	0 - 100	0 - 100
	Median	0	0	21.5	64.0

Carcasses with skin lesions obtained from the Delmarva area failed to exhibit gross lesions of leukosis in 26.5% of a total of 215 which were critically examined. The remaining carcasses of this group would have been condemned on the basis of visceral involvement. The histopathologic examination of tissue from such birds are in progress but those completed to this point indicate that microscopic evidence of leukosis is present in other organs of birds with gross skin lesions alone. With regards to gross involvement, the spleen was affected in 118 of the 215 carcasses, the liver in 105, the gonad in 61, the proventriculus in 51, the muscle in 16, the lung in 7, the heart in 6, the pancreas in 7 and the kidney in 8.

As preliminary observations at the beginning of the survey in Delmarva revealed an interesting sex distribution of carcasses exhibiting the various forms of leukosis, the sex of each condemned carcass was recorded.

1/ Calculated on individual flock basis. This accounts for failure of sum of averages to equal 100.

The data are summarized in the following table:

Sex Distribution of Carcasses with Various Lesions and Forms of Leukosis (Delmarva Area)

Form of Leukosis*	Total No. of Birds	Percent Females	Percent Males
Skin	366	85**	15
Muscle	34	62	38
Visceral	680	75**	25
Osteopetrotic	611	26	74**

An almost identical pattern of sex distribution on a smaller number of carcasses was observed from the north Georgia area.

These observations on the tendency of different forms of the disease to possess a sex predilection confirms previous reports (Brandley, C. A. et al, Amer. J. Vet. Res. 3:289-295, 1942; Burmester, B. R., Poult. Sci. 24, #5, 469-472, 1945; Burmester, B. R., and N. N. Nelson, Poult. Sci. 24, #6, 509-515, 1945; and Davis, O. S. et al, Am. J. Vet. Res. 11, #41, 428-436, 1950).

On the basis of our original observations in 1956-57, it would have been anticipated that muscle lesions would be more prevalent than the skin form. However, the results of the survey showed muscle lesions to occur only sporadically and skin lesions to be more frequent.

The fact that the avian leukosis complex is a constant but variable factor in condemnation of young chickens is exemplified by the fact that all 92 flocks in the Delmarva area and all but 1 of the 32 flocks in the north Georgia area had at least one bird condemned due to some form of the disease. In Delmarva, leukosis accounted for 50% or more of the condemnations in only 13 of 92 flocks.

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* All carcasses exhibiting skin or muscle lesions are placed in those categories irregardless of visceral involvement in the same carcass.

** Significant at 1% level by Chi square analysis.

The relative importance of the various forms of the disease can be seen in better perspective by the following comparisons of the 92 flocks in the Delmarva area:

1. Muscle lesions were observed in only 18 flocks. Muscle lesions were observed in 26% of leukosis condemned carcasses in only one flock. This was the highest incidence observed.

2. Four of the 92 flocks had skin lesions present in 50% or more of leukosis condemned carcasses.

3. Twenty-nine flocks had visceral leukosis (disregarding carcasses with skin and muscle lesions) present in 50% or more of leukosis condemned carcasses.

4. Thirty flocks had osteopetrosis present in 50% or more of leukosis condemned carcasses.

Enzyme Activity Levels in Chickens[1]/

Lois S. McDaniel[2]/

Elevations in the serum levels of several enzymes have been observed in humans and laboratory animals with various types of neoplasms. This information caused speculation about whether increased serum levels of any enzymes would occur in birds with tumors and if so, whether the altered levels could be used as a method for detecting chickens with leukosis. Besides the work done by Dr. Beard and his staff at Duke University concerning the relationships between myeloblastosis virus and adenosine triphosphatase and inosine triphosphatase activities, there is little information in the literature relative to serum enzyme levels in birds with pathological conditions.

There are two aspects to our research on enzymes. In order to obtain necessary fundamental information, experiments have been conducted on the effects of physiological stresses upon plasma enzyme levels. Chickens have been subjected to cold, frequent bleeding, and removal of feed and/or water. There is considerable individual variation in the plasma enzyme levels of normal chickens. There also appears to be considerable variation in the ability of the individual birds to withstand stress. Although statistically significant differences between the average plasma enzyme levels of the treated birds and controls have been found in some of the experiments, the ranges of the enzyme levels usually overlap. In disease studies, the enzyme levels of the affected birds would have to be beyond the range of enzyme levels in normal birds to be considered significant.

The major objective of the research is to study the effects of disease upon avian enzyme levels. Only limited data have been obtained on the effects of the leukosis complex. Some time ago a preliminary survey was made of plasma transaminase levels in chickens afflicted with various diseases. The blood samples were obtained from cases submitted to and diagnosed at the Department of Animal Pathology, University of Maine. Elevated glutamic oxalacetic transaminase levels were detected in two cases of neural, one case of ocular, and five of eight cases of visceral leukosis. No abnormal glutamic pyruvic transaminase levels were observed. In one experiment, 50 White Rock chicks were inoculated with myeloblastosis virus obtained from Dr. Burmester. Four of the 13 birds which exhibited

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1 Presented by J. F. Witter

2 Department of Animal Pathology, University of Maine, Orono, Maine

definite gross and histological lesions died before material could be collected for the enzyme assays. High glutamic oxalacetic transaminase levels, normal glutamic pyruvic transaminase levels and low lactic dehydrogenase and malic dehydrogenase levels were detected in most of the other 9 birds. There was no apparent relationship between the levels of these enzymes and the plasma levels of ATPase activity. No conclusions may be made because only a few chickens were involved in the above observations.

Large numbers of birds would probably have to be inoculated with a leukosis virus in order to obtain sufficient data, due to individual variation in the susceptibility of locally available chicks. We therefore decided to use Rous sarcoma virus (RSV) in the preliminary experiments because resistance to this virus is not as great a problem. Four experiments were conducted in which chickens were injected either subcutaneously or intramuscularly with RSV supplied by Dr. W. Ray Bryan at the National Institutes of Health. No consistent trends in the plasma levels of lactic dehydrogenase, malic dehydrogenase, glutamic oxalacetic transaminase, aldolase, isocitric dehydrogenase, or acetyl cholinesterase activities were detected.

At present we are using a RSV which Dr. Alvin Whitehill, Head of the Department of Bacteriology, University of Maine, has passed by intracoelomic injection according to the method of F. E. Popken and C. O. Boughn (J. Nat. Cancer Inst., 26, (1961): 305-313). This virus has been titrated against the NIH virus by wing web inoculation and has been found to be more virulent. Extensive involvement of the viscera has usually been obtained in 10 to 14 days by intraperitoneal inoculation of three-week-old chickens with a 10^{-3} dilution of the virus. Enzyme assays are being conducted on pancreatic tissue and plasmas of injected birds and normal controls. These studies should indicate whether differences exist between the enzyme levels of the pancreatic sarcomas and normal pancreatic tissue and if so, whether such differences are reflected in the plasma enzyme levels. The present plan includes the testing of 20 enzymes in this survey. Assay techniques which are applicable to avian tissues and plasma have been found for 14 of these enzymes. Methods for measuring the activity levels of the other six enzymes are being investigated. Although the experiments on the effects of Rous sarcomas on the activity levels of eight enzymes have been conducted, the statistical analyses of the data have not been completed.

This work is supported by PHS research grant C-4957 from the National Institutes of Health, Public Health Service.

A Dietary Factor Influencing Lymphoid Tumor of the Chicken

Carl Olson1/

About 1,300 chickens were used in controlled trials to test the influence of dietary cod-liver oil on leukosis in chickens. Four lots of cod-liver oil had no influence on erythroblastic leukosis produced by inoculation of each chicken with a relatively high dose of the infective agent.

One particular lot of cod-liver oil increased the incidence of lymphocytoma (visceral lymphomatosis) in chickens exposed to an infective agent by contact with affected chickens as well as in chickens given the infective agent by intraperitoneal injection. Another lot of cod-liver oil of essentially the same grade did not increase the incidence of lymphocytoma in chickens after they had received an intraperitoneal injection of the infective agent. The age of the birds at death from lymphocytoma was not influenced.

There was no consistent evidence of influence of cod-liver oil in the diet on other tumors, osteopetrosis or neuro-lymphomatosis.

1/ Department of Veterinary Science, University of Wisconsin Madison, Wisconsin

In-Vivo Methods for Detection of Visceral Lymphomatosis Virus and Antibody

Frank F. Piraino

This paper presents a review of procedures which are used for assaying visceral lymphomatosis (V.L.) virus and antibody in chickens and chicken embryos.

Chicken procedures utilize intravenous (I.V.) or intraperitoneal inoculations of 1-14 day old susceptible chicks. For example with the strain RPL 12 LD_{50} endpoints are based upon the erythroblastosis response which occurs in line 15I chickens within 84 days post-inoculation death (P.I.D.) and the V.L. response which occurs within 245 days P.I.D. The latter response requiring 245 days must be used for detection of small quantities of virus as is commonly the case with field materials.

Chicken embryo procedures utilize the I.V. inoculation of 11 day old susceptible chicken embryos. LD_{50} responses are based upon the development of erythroblastosis or of total neoplasia because of the additional solid tumors which occur besides erythroblastosis and visceral lymphomatosis. The early embryo response with strain RPL 12 virus occurs in less than 46 days P.I.D., or 35 days post-hatch. While no specific pathology occurs during the embryo incubation period, after hatching up to 100 percent mortality occurs within 46 days P.I.D. in high virus doses. With lower doses of virus mortality is delayed and V.L. deaths do not occur before 100 days P.I.D. For this reason embryo virus assays are most useful for studies on oncogenic spectrum, virus characterization and serology. For detection of small quantities of virus the embryo does not eliminate the need for the lengthy 245 days P.I.D. holding period. However, the early response which occurs in 46 days P.I.D. with high doses of virus is two logs more sensitive than when chickens are inoculated.

Essentially the same virus neutralization procedures are used in both chicken and embryo tests. Serum dilutions are mixed with fixed doses of virus and residual unneutralized virus measured by titration. Chicken serum neutralization titers are expressed as that dilution of serum which neutralizes two log doses of virus causing erythroblastosis and serums are considered positive for antibody by chicken embryo procedures when a particular serum dilution reduces specific mortality from 100 to 10 percent, within 46 days P.I.D.

Methods of Detection and Assay of Virus and Antibody In Vitro

John J. Solomon

While studying the Rous sarcoma virus (RSV) in vitro, where it was observed that chick embryo cells grew into foci of Rous sarcoma cells-the enumeration of which served as an accurate assay for the infectious titer of Rous sarcoma virus--Rubin (1960) found that the cell cultures from certain embryos were highly resistant to infection with Rous sarcoma virus--at least 40 times more than sensitive cultures. This was true despite the fact that the eggs were obtained from a single source. Once this resistance was encountered all subsequent transfers remained resistant to Rous virus infection.

Investigating the nature of this resistance, Rubin found that physiological changes (variations in pH, temperature, cell concentration, medium) failed to induce stable resistance to Rous sarcoma virus. Liquid medium from a resistant culture was tested by adding an aliquot to sensitive cells. After an interval of 3 to 4 days, the cells were challenged with Rous virus and were now found to be resistant. The medium of resistant cultures thus was found to contain a factor which induced resistance to Rous virus in cultures previously sensitive to this virus. This factor was called RIF, an abbreviation for "resistance inducing factor." It was present in the highest proportion in embryos obtained from a flock bred selectively for a high incidence of avian lymphomatosis and has been isolated from a number of cases of visceral lymphomatosis (V.L.) In addition, RIF like V.L. appears to be transmitted through the egg and occurs rather frequently in nature. Further evidence for the close relationship of RIF to V.L. has come from immunological studies.

This then is the basis for the in vitro assay of lymphomatosis virus and Figure 1 gives a diagrammatic representation. Sensitive cells infected with Rous sarcoma virus and plated in petri dishes show focal points of rounded up cells after 4 to 7 days incubation. These focal points, or foci, when viewed with the light microscope at a power of about 25X appear somewhat as depicted in the figure. Normal cells are restricted to growth in a monolayer. Cells infected with Rous sarcoma virus undergo a loss in contact inhibition permitting the cells to grow in several layers producing grossly visible "tumors" or foci in vitro.

Cells containing RIF, or to which RIF has been added, have a greatly reduced number of foci when challenged with RSV--as many as 10 to 50 fold fewer, or a reduction of 90 to 98%.

Figure 2 gives a schematic representation of the procedure for doing a RIF test. The first step is to obtain sensitive chick embryo fibroblasts. This is done by macerating 8 to 10 day old embryos, followed by tripsinization, collection of cells in nutrient medium, and plating of cells in large petri dishes (10^7 cells per plate). After these primary cells have grown for 3 to 4 days they are ready to be used in the assay. A primary plate is then trypsinized and the cells collected and counted. Two million cells are added to each of two small (assay) plates. Shortly after, the sample to be tested for RIF activity is also added to each plate (0.1 to 1.0 ml per plate) and the plates incubated for 3 days.

The cells are then removed from the first set of assay plates by trypsinization, collected in nutrient, counted and plated into four plates. Two plates (challenge) receive one million cells each and two (passage) get two million each. The first two plates are challenged with Rous virus (2 concentrations) and incubated for 4 days, after which the number of foci are counted. The latter two plates are incubated without challenge for 3 days and the same procedure is then followed again in order to obtain two more challenge and two more passage plates. Three days later cells from the passage plates are again collected, counted and plated; however, at this point the passaging is terminated and only two challenge plates are prepared.

Seventeen days are thus required to complete the test and twelve assay plates are used per sample tested. In addition it is necessary to have sensitive control plates. The presence of RIF is indicated by a one log (90%) or greater reduction in the number of foci when compared with a sensitive control, or a relative cell sensitivity of 0.1 or less. This relative cell sensitivity is determined by dividing the number of foci found on the test plates by the number found on the sensitive control. For example: 100 foci on the test plate and 1000 on the sensitive control would give a relative sensitivity of 100 divided by 1000 or 0.1. This would be a one log or 90% reduction in the number of foci and would be considered positive for RIF activity.

Figure 3 is a plot of a standard titration of fowl leukosis virus for RIF activity. Serial 10 fold dilutions of the stock virus were made and a RIF test run on each dilution. Twelve plates were required for each dilution to complete the three challenge results shown.

It can be seen that the end point of the titration was reached after the second challenge. The RIF titer was 10^7 interfering units per ml from the graph. Taking into consideration the initial 1:200 dilution of the tumor, the titer becomes $10^{9.3}$ interfering units

per gram of tumor, one interfering unit being contained in the highest dilution which gives evident cell resistance to Rous sarcoma virus.

In addition to determining the titer of our standard strain of RPL 12 virus, this curve can be used to estimate the potency of unknown materials. For example: a material giving complete resistance on the first challenge would be equivalent to our standard virus in titer, or up to two logs less than our standard virus. Failure to obtain resistance on the first challenge would indicate a titer at least four logs less than RPL 12, if resistance was obtained on the second or third challenge.

Comparative studies between RIF activity in tissue culture and percent total leukosis observed in chickens showed good correlation. Three tumor extract preparations which gave RIF activity also gave high total percent leukosis, while three preparations which caused low leukosis levels were negative for RIF activity. In addition tissue culture RIF titers of other virus preparations were found to agree well with titers obtained in vivo in the chick and embryo.

The in vitro procedures can also be used to measure antibody to Rous sarcoma virus or to visceral lymphomatosis virus. A diagrammatic representation of the technique is given in Figure 4. To test for RSV antibody a serum sample is incubated with Rous virus for 40 minutes at 37°C. An aliquot is then plated on sensitive cells and after 4 to 7 days incubation the number of foci are counted. A reduction in the number of foci of 90% or better is considered positive for Rous antibody. Negative sera fail to inhibit Rous virus and the number of foci is similar to that found on the sensitive control cells.

To test for lymphomatosis antibody, the serum is first incubated with enough RIF virus (RPL 12) to give complete cellular resistance to challenge RSV in the absence of antibody. The mixture is held for 40 minutes at 37°C and subsequently plated on sensitive cells. The cells are carried for 7 days under agar, trypsinized, replated and challenged with RSV. The number of foci are counted 4 to 7 days later. A positive test is indicated by the failure of the added RIF virus to induce resistance to Rous virus. An increase in cell sensitivity to RSV of 50% or greater is considered positive.

Studies on the neutralization of RPL 12 virus by a number of sera have been carried out. Serum antibody titers were obtained in vivo using chicks and embryos, and in vitro using tissue culture assays. In all cases there was good correlation between the methods. Sera which neutralized RPL 12 virus in vivo also neutralized the RIF activity of RPL 12 virus in tissue culture. This immunological evidence indicates that the RIF test is a valid procedure for the detection of viruses causing lymphomatosis.

Summary

A method has been presented for the in vitro assay of Rous sarcoma and visceral lymphomatosis viruses and antibodies to these agents. This tissue culture procedure has been shown to correlate well with procedures used in vivo. The evidence presented here and by Rubin suggest that the ability to induce resistance to Rous sarcoma virus in sensitive chick embryo cells is a property of many viruses of the avian leukosis complex. The in vitro tissue culture RIF procedure thus appears to be a rapid and sensitive method to use in avian leukosis studies.

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FIGURE 1

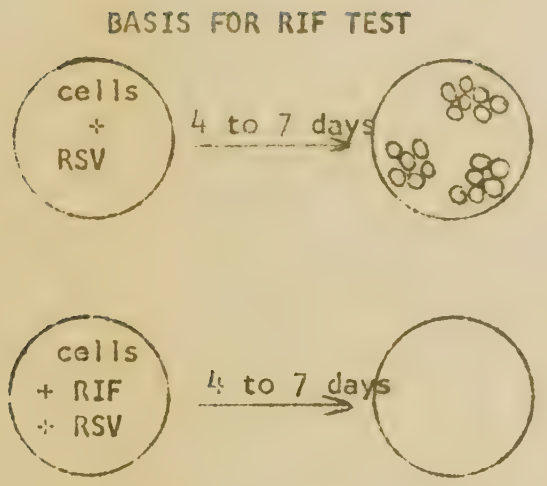

BASIS FOR RIF TEST
cells
+
RSV
4 to 7 days
cells
+ RIF
+ RSV
4 to 7 days

FIGURE 2

PROCEDURE FOR RIF TEST

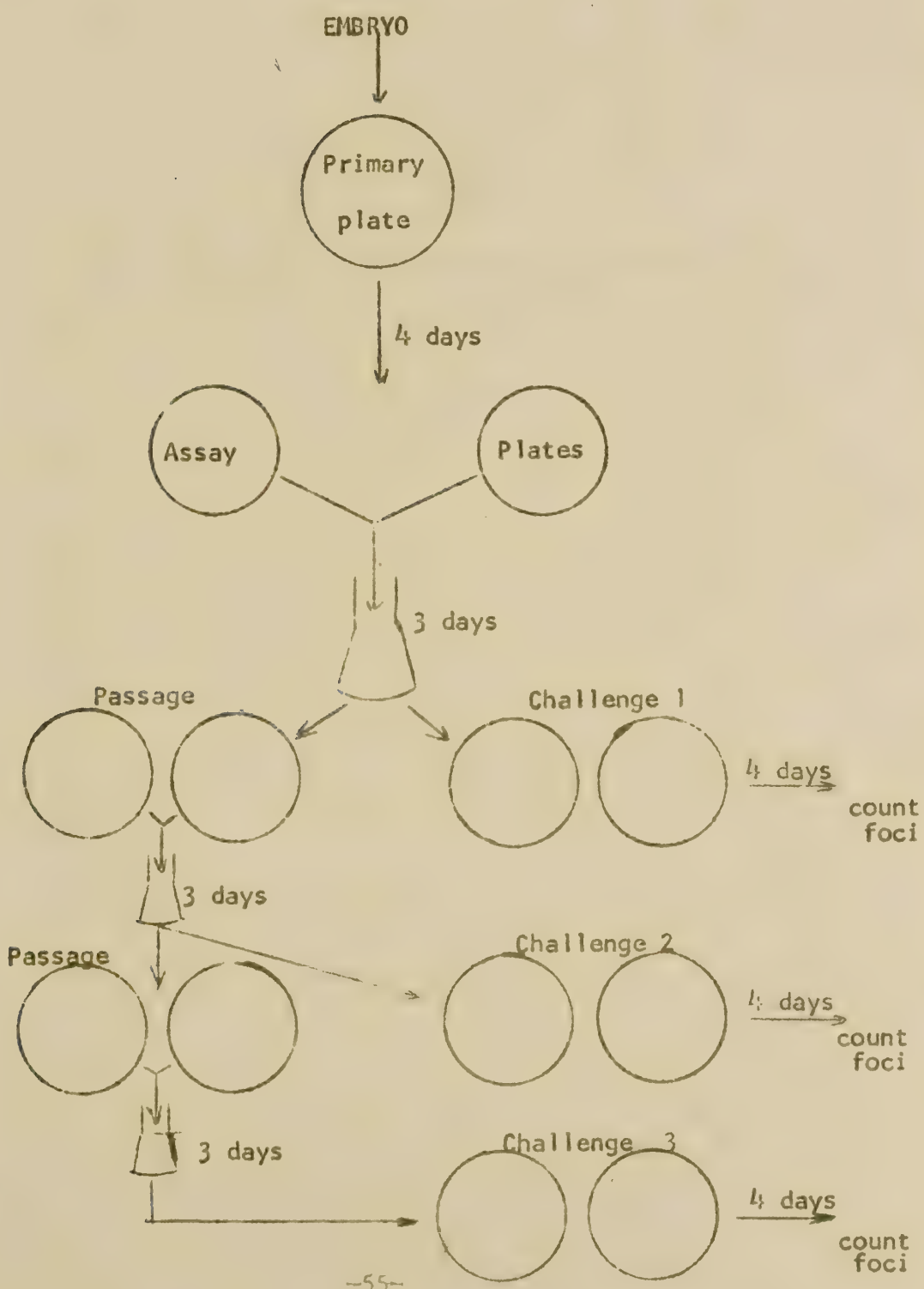


FIGURE 3

RIF Assay curve of Fowl Leukosis Virus, RPL 12, L 31

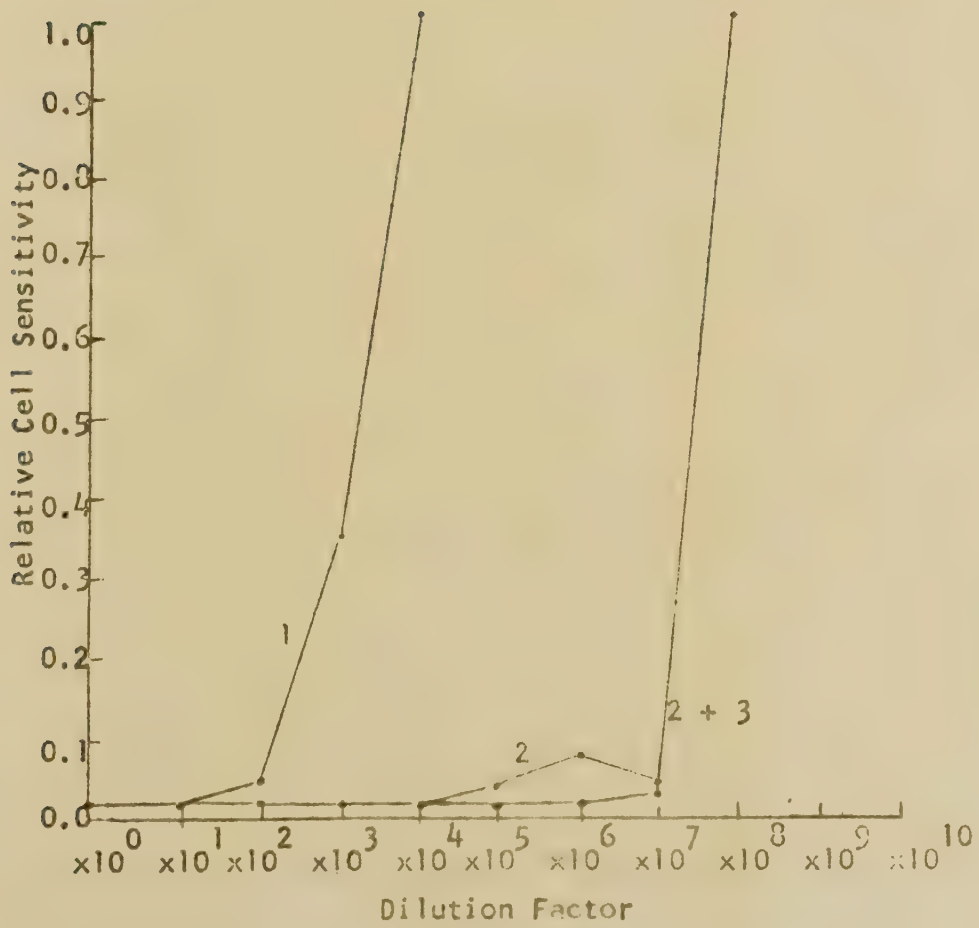
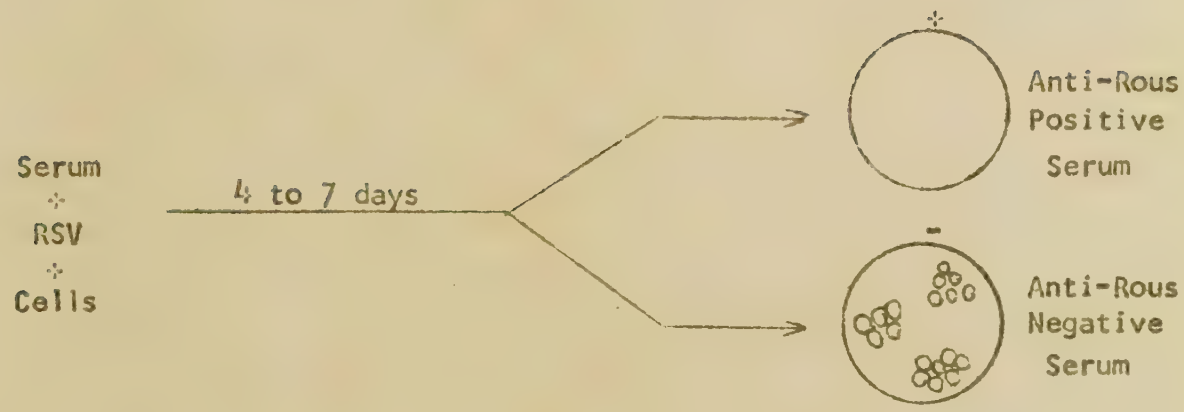
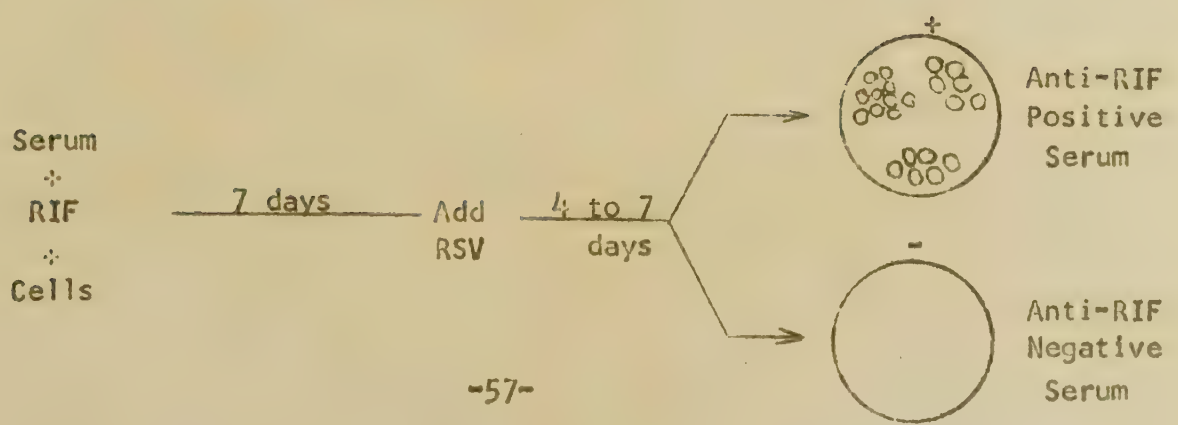


FIGURE 4

RSV Antibody Test



RIF Antibody Test



Immunity

B. R. Burmester

Progress in the development of a practical and effective immunizing agent has been slow. Actually, vaccination experiments were suspended for some time in order to isolate and study new virus strains and to develop attenuated strains. You have already heard something of these new strains and Dr. Piraino will tell you something of the serological studies that have been made with these and other strains of virus.

It seems appropriate at this time to review very briefly the results that have been obtained on the development of immunity.

In experiments on shedding of virus in the embryo and in the feces, conducted with Dr. Gentry when he was at the Regional Poultry Research Laboratory, it was found that chickens infected at an early age either by inoculation or by contact with infected chickens, an infection was acquired which remained inapparent for long periods during which time virus could be isolated from the saliva, feces, and in the eggs. However, when exposure was delayed until the chickens were adults, an active infection could not be established even though large amounts of highly virulent virus was injected intravenously into hens which were of known genetically susceptible stock and were without antibodies.

This result indicated that resistance acquired due to aging process was of sufficient magnitude to completely abort a going infection. The question was immediately asked: if the injections did not produce an inapparent infection, did they provoke an immune response? If they did, then the virus injected acted in the same way as a killed virus vaccine. Since infection was not established, there was no growth of virus and therefore the mass of virus injected was probably important in obtaining a significant response.

In our first experiment, serum and fertile eggs were obtained from 14 hens before and after they were given a series of live virus injections.

Data obtained with progeny obtained of eggs laid before and after the immunizing injection shows that there was a marked reduction in mortality in chickens of 3 groups when challenged with 3 serial 10-fold dilutions of virus.

Neutralization tests with RPL 12 virus diluted 1:10, 1:1000 or 1:100,000 when mixed with serum from immunized hens caused much less leukosis mortality than when mixed with normal serum.

Serum from immunized hens injected into susceptible chicks before being challenged with 3 doses of virus protected them from tumors, but normal serum had no effect.

In a second experiment adult chickens of line 151 were divided into 9 lots and their progeny tested for relative resistance to strain RPL 12 virus as measured by percent erythroblastosis, visceral lymphomatosis, and osteopetrosis induced. The hens were then given injections of live or killed virus and one lot left as controls.

Responses of chicks challenged with 2 doses of virus at one day from eggs laid before and after vaccination showed that there was much less of erythroblastosis, visceral lymphomatosis and osteopetrosis in chickens obtained after vaccination in comparison with those obtained before vaccination. A solid immunity was not obtained though this could hardly be expected.

Some vaccines and treatments produced chicks which were 2500 x more resistant after vaccination than before. In others the difference was only a factor of 150 x. The lots that received a heated vaccine or no vaccine continued to produce highly susceptible chicks.

Chicks from the same lots of vaccinated hens were tested a year later and their immunity was almost as high as shortly after vaccination.

Serum of hens vaccinated was collected at 4 months and at 14 months post vaccination. At both bleedings antibody levels were high and varied in proportion to the extent of immunity measured in the chicks. The control group was devoid of detectable antibodies.

The neutralization titer in the serum of day-old chicks was also found to be quite high for those of the vaccinated hens, absent in controls, but a moderate level in chicks of our naturally exposed flock.

Serums taken at 10-day intervals of hens immunized showed the passive immunity decreased rapidly so by 20 days, the level was insignificant. This is similar to that found for other viral diseases of the chicken.

A similar immunization experiment was conducted with the regular genetic population of inbred lines. Line 151 was included as positive controls and 2 vaccines A and C were tested.

Line 151 chickens showed the usual immune response. Results with vaccine C were much better than vaccine A which was a fractionated vaccine. The chickens of the regular exposed lines gave a poor immune response. This was in part due to a much too low disease

response in chickens obtained before vaccination. Thus no immune response could not have been detected in lines 6 and 9 because of the very low percent lymphomatosis before vaccination.

Leukosis mortality in chicks of lines 10 and 15 obtained after vaccination was about one-half that in those obtained before vaccination. However, the immune response was not as good as that obtained in line 151.

On the basis of our findings and those of Rubin and Hughes, it is apparent that many chickens shed virus into their eggs over long periods, especially so when they are injected as embryos, or when only a few days old. These chickens never develop antibodies, hence it is considered an immunologically tolerant infection. Since some of the chickens used in this experiment no doubt had inapparent immunologically tolerant infections, one would expect no effect of the vaccine. Likewise, some of the chickens of this population no doubt already had antibodies and even before the vaccine was injected, produced progeny with some passive immunity. Hence again one could not expect them to show an immune response following vaccination.

It became quite obvious that in order to test out the effectiveness of a vaccine, hens and chicks of known infection-antibody status must be used. Furthermore, the present data indicate that vaccination alone will not eliminate the carrier hen which lays infected eggs, thereby reproducing the tolerant infected chicken.

Two exploratory experiments suggest that direct immunization of young chickens may be possible.

Three lots of chicks 2 days of age were given a very small dose of live virus--a -7.0 log dose--then at 10-day intervals, together with other chicks of the same age not previously injected, were given a large dose of virus. The results show that chickens given the 2nd dose at 22 and 42 days of age were much more resistant to the 2nd large dose of virus than the controls or when the interval was only 10 days.

The requirement of a time interval suggests that the increased resistance was due to an immune response rather than an interference phenomena.

To further test out this idea, a second experiment was conducted wherein live virus or formalized virus was given by different routes at 5 and 16 days of age. The formalized virus had no effect. The live virus given subcutaneously produced the greatest increase in resistance to challenge at 26 days of age.

It is of interest to mention here that we have one field isolate or virus strain which has caused very little pathology in our susceptible line 15I chickens, yet has provoked a high antibody level when chickens were inoculated at 1 day.

Further experiments must be conducted to fully test out the possibility of immunizing young chickens with killed and attenuated vaccines.

Leukosis Virus and Antibody[1]

W. F. Hughes[2]

In the spring of 1960 Dr. Harry Rubin of the University of California found a virus in chick embryos which rendered tissue culture cells resistant to Rous sarcoma virus infection (1). This virus proved to be indistinguishable from avian leukosis virus and led to the development of the interference technique as a test for leukosis virus. The close immunologic relationship of leukosis virus and Rous virus provided a companion technique for antibody assay.

These techniques were applied to sera from a strain of White Leghorn chickens selected at Kimber Farms for a high incidence of lymphomatosis. The detection of both antibody and virus in this strain led to its use in epidemiological studies.

Because the initial isolation of leukosis virus was from embryos, attention was centered on a mechanism of infection of the embryo. In an earlier study on the pattern of congenital transmission (2) it was found that adult birds of this population could be divided into two major classes with regard to presence of leukosis virus or antibodies in the blood. The first class was composed of birds which had a persistently high leukosis viremia over the 10-month period of investigation but failed to develop any evidence of circulating antibody at any time. Birds of the second class had no detectable viremia at any time during the course of the experiment, but had antibody to leukosis virus in variable concentration.

The present investigation amplifies and extends the study of general transmission of leukosis virus by considering the following questions:

1. Does congenital infection of a bird with leukosis virus lead to persistent viremia and absence of anti-leukosis antibody (immunological tolerance?) in that bird?

2. How do the birds which are free from leukosis virus at hatching respond to contact with congenitally infected birds? Do they develop viremia? Do they develop antibodies, and if so, when, and to what degree?

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1 This article is a condensation of the paper "Tolerance and immunity in chickens following congenital and contact infection with visceral lymphomatosis virus" by H. Rubin, L. Fanshier, A. Cornelius and W. F. Hughes (Virology, May 1962).

2 Kimber Farms, Fremont, California

3. Does pattern of congenital transmission disclosed by the study of parents and progeny apply to a third generation?

The plan of study was to characterize the viremic and antibody status of some 800 progeny of 75 hens and 10 roosters by repeatedly bleeding the birds and testing the sera for virus and antibody.

Materials and Methods

Strains of Chickens

The Kimber Farms Strain 13 single comb White Leghorn flock was used as a source of birds throughout this study. This strain has been bred selectively for a high incidence of neoplasm for over 10 years. Ten single male pedigree pens were set up with 75 hens distributed amongst them. Only 63 of the hens in the present study produced enough viable progeny to permit their classification as transmitters or non-transmitters of the virus.

Eggs were obtained by trapnesting of hens and the chicks were wing-banded at hatching for familial identification. The chicks were reared in an isolated litter house. Seven hatches were obtained and both congenitally infected and non-infected birds in each hatch were reared together to simulate the conditions of exposure commonly encountered in commercial flocks.

Virus Assay

Leukosis viremia and antibody were detected by the techniques of Rubin (2).

In an earlier report it was shown that Rous sarcoma virus neutralizing activity of a serum is a useful gauge of its anti-leukosis activity (1). This, of course, follows from the fact that Rous sarcoma virus and leukosis virus are immunologically related. In the earlier study and correlation coefficient between the anti-leukosis and the anti-Rous sarcoma virus activity of various parental sera was found to be greater than 0.8. During the course of the present study a comparison of the anti-Rous and the anti-leukosis activities of progeny sera was made, and an even stronger correlation was found (Table 1). Since the tests for neutralizing activity against Rous sarcoma virus were far easier to quantify than the test against leukosis virus, all the sera were tested for anti-Rous activity and only selected sera were tested for anti-leukosis activity as well.

Results

Antibody and Viremia in the Sera of Parental Birds

Thirteen of the breeding hens and five of the roosters were heavily viremic. Only two of the 18 heavily viremic birds had antibodies to Rous sarcoma virus and none had antibodies to Leukosis virus.

Three birds had low titers of leukosis viremia. Antibody to Rous sarcoma virus was found in two of these three sera, they were not tested for the presence of antibody to leukosis virus.

The remaining 64 adult birds whose sera were tested had no detectable viremia but in marked contrast to the viremic birds, 60 had antibodies to Rous sarcoma virus in at least one bleeding and 57 in all bleedings. There were five birds with no detectable antibody to Rous sarcoma virus but each of these had low titered antibody to leukosis virus.

As in the earlier and more limited study (2) the adult population could be classified into two groups: a) the heavily viremic birds without antibody to leukosis comprising about 1/5 of the population, and b) the non-viremic birds with antibody to leukosis comprising most of the remaining 4/5 of the population. As noted above there is a third minor class of birds with low-level viremia. Although two of the three birds in this class had antibody the sample was not large enough to determine whether it is representative of the class.

Antibody and Viremia in the Sera of Progeny Birds

In the earlier report on congenital transmission of leukosis (2) it was found that certain hens consistently produced infected embryos (Table 2). Chicks were bled at various times up to seven weeks after hatching to determine whether chicks derived from infected embryos continued to produce virus after hatching. It was found that hens which produce infected embryos also produced chicks which continued to maintain heavy viremia. It can, therefore, be concluded that the cells of congenitally infected birds continue to produce virus after hatching and that the resulting heavy viremia in young chicks is an adequate indicator for congenital infection in a survey such as the present one (Table 3). Once this was established, the collection of sera from the progeny of all parental birds was begun so that on the one hand the progeny could be individually identified as congenitally infected with leukosis virus or not, and on the other hand each parent could be classified as a persistent congenital shedder or a non-transmitter of the virus. Because of the large numbers of progeny birds involved, and the limited numbers of sera which could be conveniently tested for leukosis virus and antibody, the progeny were bled in staggered groups, i.e., one hatch was bled at two weeks of age, another at four weeks, another at six weeks and so on. At 13 and 14 weeks a second bleeding of the first bled group

was carried out, and in subsequent weeks second bleedings were obtained in turn from the remaining hatches. One hatch of about 100 birds was bled four times to obtain multiple records on individual birds. The last bleeding was carried out at 29 weeks. By this time the birds had reached full reproductive maturity.

Only 63 of the original 75 hens produced enough chicks to permit classification with regard to congenital transmission. Eighteen of these 63 produced more than one chick with a heavy viremia and are classified as persistent congenital transmitters. Ten of the 18 congenitally transmitting hens were viremic; eight were non-viremic and had antibodies to leukosis virus. (All the fertile viremic hens were congenital transmitters, whereas only about one in seven of the non-viremic birds were.)

The remaining 45 fertile hens produced 545 chicks, the vast majority of which had no viremia or low level viremias. Nine of the 45 hens had single chicks with a high viremia on the first breeding but for reasons discussed below it is likely that most of these chicks became viremic by contact rather than congenital infection.

In this study there were 212 congenitally infected and 716 non-in infected progeny, giving a ratio of 1 congenitally infected bird for every 3.4 non-infected birds in the strain 13 progeny population.

Figure 1 illustrates the viremic status of the progeny as determined from staggered bleedings of the various hatches. The progeny of the non-transmitting hens are represented in the upper half of the figure and progeny of transmitting hens are presented in the lower half of the figure. All hatches of the virus infected families contained 80-100% heavily viremic birds in all bleedings between 2 and 29 weeks of age. In contrast to this group only very few of the progeny in the non-shedding families had any viremia up to seven weeks of age, and even these few birds had, in the main, low level viremias. The proportion with the detectable viremia in this class increased to about 25% at nine weeks and to about 45% at 14 weeks. After 14 weeks proportion with detectable viremia decreased to a constant level of about 10%. The progeny of non-transmitters and transmitters were brooded together and it is assumed that the virus spread from the latter to the former.

The viremia found in the progeny of non-transmitters were mainly of low level, with only about 5% developing heavy viremias. Many of these chicks had no virus at an earlier bleeding and were, therefore, not congenitally infected. This indicates that a heavy persistent viremia can occasionally result from contact infection in the Strain 13 flock. It also indicates that sporadic congenital transmission was rare among the group of hens classified as non-transmitters in this study.

The Rous sarcoma virus antibody status of the progeny population as a function of time after hatching is shown in Figure 2. It can be seen that only small proportion of the progeny of transmitting families developed antibodies to Rous sarcoma virus. Study of successive bleedings of individual chicks in transmitting families showed that antibody developed only in those few progeny which did not exhibit the early heavy viremia indicative of congenital transmission.

Antibodies could be found in 34% of the progeny of the non-transmitting hens at two weeks after hatching. This was to be expected in view of the high proportion of hens with antibody and the fact that antibodies in birds are transferred with more than a tenfold decrease in titer from hen to embryo through the yolk. The passively transferred antibodies could no longer be detected in the four-week bleeding. Actively produced antibodies appeared in a small proportion of the birds at nine weeks. The sharpest increase in the proportion of birds with antibody occurred between 14 and 18 weeks. It was during this time interval that there was a marked decrease in the proportion of birds in this group with detectable viremia, and it is likely that the decrease was a direct consequence of the immunological reaction of the host to the infection.

Between 18 and 29 weeks there was a slow increase from 80% to 90% in the proportion of contact infected birds with antibody. More than half of the remaining 10% of the contact infected population was accounted for by the birds which developed a high viremia with no antibodies, similar to that found in congenitally infected birds. Less than 5% had neither detectable antibodies to Rous virus or leukosis viremia. These birds had either escaped infection entirely or had too low a titer of antibodies for detection.

Congenital Transmission of Virus by Males

There was no indication of successful congenital transmission by viremic roosters. In contrast to the obvious role of viremic hens in congenital transmission of leukosis virus, the viremic rooster appears to be unable to transmit the virus to their progeny at fertilization.

Discussion

The basic pattern for congenital transmission of leukosis virus has been confirmed through three generations in a single flock of White Leghorn hens. It was found that viremic females are consistent congenital transmitters of the virus while only about one in seven females without viremia congenitally infects her offspring and these

with less consistency than the viremic females (Table 3). There is no evidence for congenital transmission by the male, and it must be considered likely that the viral genome is lost along with the shedding of RNA which occurs in spermatogenesis. There is no doubt, however, that the viremic male would infect susceptible females housed in contact.

The congenital transmission of leukosis virus by non-viremic females with substantial antibody titers has been demonstrated. It is perfectly clear that virus multiplication can persist indefinitely in the germ cells of the hen in the face of high antibody titers in the circulating blood.

Perhaps the most striking finding of this study is the demonstration that congenital infection with leukosis virus leads to immunological tolerance of the virus. The congenitally infected chicken continues to release large amounts of virus into the circulation for at least seven months and probably for the full life of the chicken, as judged by persistently high viremia seen in adult birds tested between 12 and 22 months of age. That the tolerance is specific for leukosis virus and is not due to a generalized breakdown of immunological competence is indicated by the finding that leukosis viremics can be induced to form antibody to influenza virus as readily as non-viremic (Levinson and Rubin, unpublished).

The tolerantly infected bird is an ideal vehicle for the dissemination of virus. With a high proportion of its cells actively producing virus and without the deterrants of antibodies it is likely that high concentrations of virus are present in all bodily secretions and excretions. Non-viremic chicks in contact will no doubt become infected as soon as passive protection from the hen disappears.

Since all the birds in the flock regularly become infected with leukosis virus it is evident that visceral leukosis follows infection in only a small proportion of cases. This holds true even among the congenitally infected birds in which the virus multiplies steadily from embryonic life onward.

Summary

The congenital transmission of a field strain of visceral lymphomatosis virus has been shown to be matroclinous. All viremic hens and one out of every seven non-viremic hens in a flock selected for high incidence of neoplasm were persistent congenital transmitters of the virus. Congenital infection resulted in the establishment of immunological tolerance to the virus and the birds made no detectable antibody to the virus even though they were fully competent to produce antibody to influenza virus.

Non-infected birds became infected by contact with congenitally infected birds. They exhibited a transient viremia which was usually of a low level. The viremia was followed by antibody production which cleared the blood of detectable virus.

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Table I

Correspondence between Antibodies to RSV and RIF in Progeny Sera

Age of Chickens	Sera of Chickens with Antibodies to RSV: Antibodies to RIF	Sera of Chickens with Antibodies to RSV: No Antibodies to RIF	Sera of Chickens without Antibodies to RSV: Antibodies to RIF	Sera of Chickens without Antibodies to RSV: No Antibodies to RIF
2-4 weeks	10	0	0	19
7 weeks	--	-	0	25
9 weeks	2	0	0	16
13 weeks	3	0	0	15
18 weeks*	none tested	none tested	0	13

* Sera were selected for the Anti-RIF test only from among those with no antibody to RSV.

Table 2

Correlation of Heavy Viremia in Chicks with Congenital Infection

	Hen No.	Infected embryos*	Chicks under 7 weeks of age with heavy viremia
Congenitally	1	16/17	16/16
Transmitting	3	5/9	7/13
Hens	9	14/15	9/9
	19	9/9	1/1
		44/50	33/39
Non-transmit-	2	0/20	0/14
ting Hens	4	0/14	0/18
	5	0/15	0/6
	8	0/8	0/11
	10	0/12	0/19
	12	1/11	0/10
	13	0/16	0/8
	14	0/20	0/17
	18	0/15	0/5
	20	0/17	0/10
		0/148	0/118

* From Rubin et al., 1961

Table 3

Congenital Transmission by Viremic and Non-Viremic Hens as Detected by Viremia in Chicks

Hen No.	Heavy Viremia in Progeny of Viremic Hens	Hen No.	Heavy Viremia in Progeny of Non-Viremic Hens**
1	15/15*	41	3/11
3	7/13	45	7/13
9	9/9	46	7/13
43	15/15	60	17/19
57[a]	11/11	61	9/10
62	13/13	74	6/9
63	4/5	81	6/8
67	7/7	82	3/4
69	5/5	Total	58/87 = 67%
70	14/14		
Total	100/107 = 93.5%		

* Number of heavily viremic progeny total progeny

** 545 progeny tested from the remaining 45 non-viremic hens were themselves non-viremic

a weak viremia in hen

Serological Studies of Avian Tumor Viruses

Frank F. Piraino

Serological tests were done in embryos and tissue culture. Only the Rous sarcoma virus (RSV) and strain RPL 12 of visceral lymphomatosis (V.L.) were studied in tissue culture; other virus strains were studied almost exclusively in chicken embryos. These studies involved the RPL 12 strain of V.L., the RSV, and two recent field isolates passaged three times in line 15I chickens. They are RPL 26, a virus isolated from an outbreak flock near Akron, Ohio; and RPL 29, a virus isolated from a similar flock near Petoskey, Michigan.

In addition to the hyper-immune serums of the above named viruses, other serums used were from a chicken which survived an outbreak in Georgia designated as Lackaby, and a serum from a chicken exposed to natural infection in California designated Hughes #1. Hyper-immune serums of RPL 12, RSV, and RPL 29 viruses were obtained by repeated injections of line 15I chickens. From checkerboard tests of each serum against each virus strain several tentative conclusions were drawn.

1. Fowl leukosis viruses causing field infections are antigenically more complicated than the RPL 12 and RSV laboratory adapted strains.

2. Considerable antigenic variation exists among leukosis viruses.

3. Viruses having similar oncogenic spectrums as determined by chicken and embryo inoculation tests, may be quite dissimilar antigenically.

Additional studies comparing serum neutralizing activity by chicken, embryo and tissue culture procedures indicate that tissue culture tests are at least as sensitive as the in-vivo virus neutralization procedures. Also, results obtained from 152 individual tests in tissue cultures indicate that antibody to RSV is an excellent indicator for the presence of V.L. antibody, thereby avoiding the use of the more difficult RIF virus neutralization test.

The Development of a Rapid Serological Test

William Okazaki

Abstract

The difficulties encountered in attempting to adapt conventional simple and rapid *in vitro* test procedures to the avian tumor virus-antibody system appears to be associated to some extent to the concentration of viral antigen obtainable using conventional methods of preparation. The complement-fixation test presumably should be a satisfactory test for detecting antibody in a suspect serum. However, it was found earlier (1, 2, 3) that both heated and unheated chicken sera were anticomplementary or incompatible with guinea pig complement using conventional techniques.

Recently a group of workers in Minnesota (4, 5, 6) have elucidated the role of guinea pig complement using the avian antiserum-antigen system and reported considerable success in detecting complement-fixing antibody with various avian disease viruses.

Recent studies at the Regional Poultry Research Laboratory in East Lansing, Michigan have shown that with slight modifications of Brumfield's modified complement fixation technique (6) complement-fixing activity of anti-Rous virus serum can be detected. Although the sensitivity of the test is considerably less than the *in vitro* tissue culture and *in vivo* neutralization tests, the correlation obtained is excellent.

Studies are now in progress to standardize the reagents for adapting the test to other avian tumor antigen-antibody systems.

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Fluorescent Antibody Techniques

H. Graham Purchase

Albert H. Coons as a second year medical student in 1934, working under John Enders at Harvard, originally started to trace the course of antibodies before and after anaphylactic shock in the guinea pig by means of the precipitin reaction. Later he turned his attention to other hypersensitive reactions. He assumed it must be easier to locate antigen than antibody and he tried to get a visible micro-precipitate. Here he struck the idea of a colored molecule. The first attempt to label antibody was by diazotisation. Marrack in 1934 first labeled anti-cholera and anti-typhoid antibodies with tetra-azotised benzidine resulting in a red stained active antibody. It was easy to show that clumped organisms were specifically reddened. However, under the microscope the organisms showed up only faintly pink and it was obvious that small quantities of antigen could not be detected by this method.

The first attempt by Coons using fluorescent dyes was to couple anthracene isocyanate to anti-pneumococcal antiserum. This preparation agglutinated pneumococci and under ultraviolet light the organisms were brilliantly fluorescent. The first successful coupling of fluorescein isocyanate to antibody was in 1942.

About 1947 the first organ suspension was used to remove some of the nonspecific fluorescence. Since that time the techniques for purifying and labeling of antibodies have advanced.

The first prerequisite for successful staining is a very high titred antiserum. From this the gamma-globulin fraction containing the antibodies must be extracted either by precipitation with ammonium sulphate or alcohol, or using a suitable ion exchange resin. The purified gamma-globulin must then be conjugated with the dye. The first dye to be used was anthracene isocyanate but this was soon superseded by fluorescein isocyanate. Fluorescein isothiocyanate is much easier to work with and gave better results especially when adsorbed onto filter paper or more recently onto celite or diatomaceous earth. Rhodamine B, nuclear fast red, tetra-methyl rhodamine and other dyes have been used. Any unconjugated fluorescein must be removed by dialysis or gel filtration. Finally some of the nonspecific antibodies must be removed by adsorption with tissue powders.

The labeled antiserum is laid over the preparation to be stained and the excess is washed off after a suitable time and the preparation is examined under an ultraviolet microscope. This is the direct or one stage method of staining. In the indirect or two stage method

the antigen is first allowed to react with an unlabeled specific gamma-globulin. This forms an antigen-gamma-globulin complex. Using a labeled anti-gamma-globulin it is possible to demonstrate this complex. The advantage of this method is that only one labeled serum is necessary for a number of different antibody-antigen reactions. Thus using a labeled anti-chicken gamma-globulin prepared in rabbits one could show up the antigen in Infectious Bronchitis (using the relevant antiserum) and, for instance, Rous sarcoma virus.

The third method is the complement staining method. Here one allows the antigen, the unlabeled specific antibody and complement to react. They form a complex which can be made visible using labeled anti-complement.

Antigen can be shown up by any of these methods and the test is known to be very specific. In some cases it is necessary to fix the antigen in which case a method must be chosen which does not destroy the antigenicity of the preparation. For Rous sarcoma virus the usual method is immersion for 10 minutes in dry acetone at -60°F. Unfortunately when cells infected with RPL 12 were fixed by this method the antigenicity was lost so to date only unfixed preparations have been stained successfully here.

Vogt and Rubin in California have clearly shown the stages in infection and production of Rous sarcoma virus in chick embryo fibroblast tissue cultures. The viral antigen is first visible on the second day after infection. On the third day the number of fluorescing cells has increased and the cells show a bright fluorescence at the cell surface and later in the cytoplasm. The cells lose their fibroblastic appearance and become rounded and at the same time detachment of the antigen from the surface occurs. It is expected that the antigen of leukosis viruses will also show up first at the cell surface and then in the cytoplasm.

The fluorescent antibody technique is fairly new in this Laboratory and the first indication of its use is to show up the antigen, probably of RIF, in resistant primary cultures of chicken embryo fibroblasts as early as 3 days after explantation. These results have not yet been confirmed. If it is possible to show up small quantities of antigen in tissue culture then it may help considerably in eliminating the time necessary for challenging with Rous sarcoma virus.

On the other hand, fluorescent staining has unlimited value as a tool for searching for antigen in histological sections. It may help locate what happens to the leukosis virus between the time that the bird becomes infected and develops visceral lymphomatosis--an incubation period which is known to be longer than a year in some cases.

At present we have a large project under way for the production of hyperimmune antisera. These have to be titrated in tissue culture and the suitable ones will be labeled.

There are some very important limitations to the technique. In order that a negative result mean anything the test must be carefully controlled especially with regard to fixation of antigen. Undesired staining reactions can usually be dealt with by treatment of the labeled antiserum with acetone dried tissue powder or by dilution of the serum. A more difficult problem is defining the specificity of the serum especially when very small quantities of the unknown antigen are present. Confusion may also arise from the staining of a closely related antigen or one to which the serum incidentally has antibodies.

Despite these limitations the fluorescent antibody technique is extremely useful in virus research and I am sure it will prove useful in cancer research here.

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Contributions of the Phenomena of Avian Virus to Cancer Research

J. W. Beard

(With the permission of Mr. Don Turnbull, we are quoting the report he wrote of Dr. Beard's talk. This appeared in the May issue of THE AMERICAN POULTRY & HATCHERY NEWS.)

"Declaring that research workers in poultry are sitting on a ton of gold in virus tumor research, Dr. J. W. Beard, M. D., Director of the School of Surgery, Duke University, told an audience of 150 research men at the Avian Leukosis Conference in East Lansing, Mich., April 25-26 that the fundamental problem has resolved itself to one in biochemistry and that the need now is for a greater concentration of effort on the main problem.

Dr. Beard said a few million dollars properly spent now would enable the poultry industry and others in cancer research to study all phases at once.

Long interested in the study of Avian Leukosis as a tool helping to solve the mysteries of human cancer, Dr. Beard told his audience that the "chicken is the unit offering the most fruitful field for earliest results". His words offered encouragement to industry scientists dealing with the baffling complex.

The problem is that of the interrelationship between virus and cell and molecular development thereof, in Beard's opinion.

More Look to Chicken

Beard charged that work on cancer has been delayed because of the unwillingness to recognize that the virus tumor in chickens offers an excellent tool for studies on the cancer problem in general.

"It is best suited for concentrated, organized effort," the scientist opined.

He paid tribute to the work of Dr. B. R. Burmester, Dr. Nelson Waters and others at the Federal Regional Poultry Disease Laboratory, sponsors of the two-day meet, for their work on oncogenic (cancer-inducing) virus spectrum variations. The laboratory discovery that host response often spells the difference in virus performance has been a milestone in the study of cancer."

Viruses and Parthenogenesis in Turkeys

M. W. Olsen[1]/

Spontaneously occurring parthenogenesis in unfertilized eggs of the domestic turkey was first noted in Beltsville in 1952. Unselected Beltsville Small White hens, as virgins, produced eggs of which approximately 16 percent showed parthenogenetic development following an incubation period of 8-10 days. The development encountered consisted chiefly of a thickened sheet of unorganized cells which covered varying areas of the yolk surface. Seldom was blood encountered and only on rare occasion, an embryo. Since the initiation of a selective breeding program in 1954, there have been significant yearly increases in the level of parthenogenesis, the extent of which is evident from data presented in Tables 1 and 2. By means of selection, a strain of Beltsville Small White turkeys has been developed whose unfertilized eggs show a high incidence of parthenogenesis. Over 40% of the unfertilized eggs from this parthenogenetic strain of turkeys can be expected to show some degree of development on being incubated. Parthenogenetic embryos can be expected in approximately 10-12% of the eggs. Some of these embryos will survive to hatching, a few emerging unaided from the shells. All parthenogenetic poults thus far encountered have been diploid males. Some of these males have been raised to maturity, and a few have produced viable sperm. Semen from parthenogenetic males has been used to inseminate virgin turkey hens resulting in fertile eggs from which normal poults (both male and female) have been hatched.

The exact nature of the stimulus responsible for this asexual type of development remains obscure. Some data would indicate that inheritance plays a decisive role, while other data seem to point to some environmental factor as also associated with parthenogenesis. This naturally has led to much speculation regarding the nature and mode of action of the stimulus involved.

Those who favor the genetic concept point to yearly increases in parthenogenesis since the initiation of the selective breeding program, particularly the increases in the categories of blood and of embryo formation.

It should be pointed out, however, that a change was also made in the vaccination program in 1955. Prior to this date, turkey hens on experiment received only one routine vaccination for fowl pox. This

1 Poultry Research Branch, ARS, Animal Husbandry Research Division, Beltsville, Maryland

vaccination was given to poults when they were only 6-8 weeks of age. Since 1955, a booster shot of live fowl pox has been given to turkey hens at the time of sexual maturity (approximately 30-40 weeks of age). This change in the vaccination program should also be considered as a factor responsible for the increased incidence of parthenogenesis, though adequate controls are lacking in these tests. Experiments in progress, however, will compare the effects of selection with and without fowl pox vaccination.

Data presented in 1956 (1) and in 1962 (2) show a direct relationship between live fowl pox virus and the level of parthenogenesis in chicken and turkey eggs. In both studies significant increases were noted in the level of parthenogenetic development in unfertilized eggs after hens had been vaccinated.

Live Rous sarcoma (3) and Newcastle disease viruses (4) likewise brought about an increase in the level of parthenogenetic development in unfertilized eggs. Significant increases in the incidence of parthenogenesis were also noted in unfertilized eggs of White Leghorn chickens following a natural outbreak of visceral lymphomatosis (5).

Viruses appear to differ in their ability to bring about an organized type of parthenogenetic development. Live fowl pox virus has been the only one of the four viruses tested thus far which appears to be associated with increases in blood and embryo formation.

Viruses, to be effective as parthenogenetic inducing agents, must be in an active state. No increase in the level of parthenogenesis could be detected in turkey eggs after unvaccinated turkey hens had been inoculated with beta-propriolactone inactivated fowl pox, Rous sarcoma or Newcastle disease viruses (6).

Summary

A strain of Beltsville Small White turkeys has been developed whose eggs show a relatively high incidence of parthenogenetic development. This tendency toward an asexual type of development was in large part the outcome of a selective breeding program initiated in 1954. A twice yearly vaccination program for fowl pox is also considered to be associated with the development of this strain of birds.

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Table 1

August, 1960

Incidence of Parthenogenetic Development in Unfertilized Eggs of Beltsville Small White Turkeys

Year	No. of hens	No. of eggs tested	Number and percentage of eggs found to contain							
			Membranes only		Blood and membranes		Embryos		All categories of parthenogenesis	
			(No.)	(%)*	(No.)	(%)	(No.)	(%)	(No.)	(%)
1952	29	934	156	16.7	0		2	0.2	158	16.9
1953	23	1463	191	13.1	12	0.3	3	0.2	206	14.1
1954	103	5930	1198	20.2	71	1.2	44	0.74	1313	22.1
1955	116	5754	1251	21.7	161	2.8	84	1.4	1496	26.0
1956	120	6013	1341	23.6	198	3.3	251	4.2	1330	30.5
1957	161	8808	2293	26.6	490	5.7	360	4.2	3143	36.5
1958	214	8045	2060	25.6	492	6.1	722	9.0	3274	40.7
1959	78	2926	718	24.5	153	5.3	342	11.7	1213	41.5
1960	60	2815	720	25.6	123	4.4	277	9.8	1120	39.8
1961	161	10060	2366	23.5	957	9.5	1249	12.4	4572	45.4

* Percentages are on basis of total eggs tested.

Table 2

August, 1960

Number and Percentage of Parthenogenetic Embryos Encountered Each Year and the Size Each Attained in Relation to that of a Normal Turkey Embryo

Year	Total no. embryos	Stage of development - days							
		2-10		11-20		21-28		Hatched	
		(No.)	(%)	(No.)	(%)	(No.)	(%)	(No.)	(%)
1952	2	2	100.0						
1953	3	3	100.0						
1954	44	37	84.1	2	4.5	4	9.0	1	2.3
1955	83	70	84.3	5	6.1	8	9.6	0	0.0
1956	252	166	65.9	19	7.5	59	23.4	8	3.2
1957	360	249	69.2	28	7.8	71	19.7	12	3.3
1958	721	492	68.3	75	10.4	134	18.5	20	2.8
1959	342	209	61.1	36	10.5	85	24.9	12	3.5
1960	277	155	56.0	20	7.2	87	31.4	15	5.4
1961	1249	744	59.6	85	6.8	314	25.1	106	8.5

Dr. King asked representatives of commercial breeder organizations to comment on their efforts to increase exposure to lymphomatosis by artificial means.

Dr. Shrode - DeKalb: Our objective is to produce profitable chickens. The big problem is to expose birds at known levels. We are compelled to do all we can to select for general livability and hope that we are successful in improving resistance to many things. Livability is a compound, complex character. Our test chickens have very low mortality, therefore, we cannot do much selection for livability.

One result is that the progeny find themselves in circumstances that their ancestors never found themselves. We hope that buffering due to crossing is sufficient to let them survive in the situations they will encounter in the field.

Dr. Hughes - Kimber: There have been a number of projects undertaken along this line. It took only a few years of work to get the leukosis level to the point where selection was not effective.

We want to know how to artificially expose progeny.

1. We reared baby chicks next to old hens. This failed to the extent that they got less leukosis than controls.

2. We inoculated chicks with rapid passage tumors and allowed them to die in the pens where replacement chicks were being raised. This also failed.

3. More recently, we have been taking rapid passage tumor material and inoculating dams and checking progeny. When they were reared and met natural exposure there was no mortality difference in progeny of inoculated dams and controls.

We keep asking where we go next for an exposure that will approximate a natural exposure.

Wade Smith - Blanton Smith: We had the same difficulty of trying to find differences that didn't exist.

We approached it differently. We made test matings on a number of different poultry farms throughout the country rather than creating the exposure artificially. We tried to go where natural exposure existed. Our birds usually lived when placed where many of the birds had died there before. We traced back to hatch and variety, visited farms of every customer who got the same hatch and variety.

Apparently not a simple cause and effect relationship is involved, but some kind of post-hatch environmental reason.

The Genetic Approach to Research on Lymphomatosis

Lyman 3. Crittenden

The genetics work of this Laboratory is divided into two phases. The aim of the first is to supply genetic information of practical value to the breeder and to carry on basic studies on the inheritance of resistance to viral neoplasms. The aim of the second is to supply genetically uniform susceptible or resistant chickens for the other experimental work of the Laboratory. I will outline these phases of work by pointing out methods which can be used and the unique material and research opportunities available at this Laboratory.

Phase I

The first step in the genetic analysis of any trait is to establish a hereditary basis for some of the variation in that trait. The three major sources of variation thought to affect mortality from visceral lymphomatosis are genetic, non-genetic maternal and external environmental factors.

The male parent as far as we know, contributes only chromosomal genetic material to the chick while the female parent contributes virus, antibody and other non-chromosomal as well as chromosomal material. The immediate environment in terms of exposure and other external environmental factors certainly has a large influence.

Any one of these sources of variation may be studied in each of three general ways. The first is to ignore the other two; the second is to completely control the other two which is usually impossible and always time-consuming and expensive; and the third is to design experiments so that the other two sources do not bias the results. The third method is usually the one of choice.

Since the male influence may be considered to be entirely genetic the geneticist is able to design experiments to study hereditary variation free of bias from other sources. As you have seen, Dr. Waters has consistently switched males in his experiments to demonstrate an effect on the resistance of progeny from particular females. Another design used by geneticists dealing with inbred lines is the diallel cross. Figure 1 illustrates a series of crosses which we plan to make this next year. This involves mating four of our lines in all combinations, giving 16 types of matings which we feel will give a wide range of resistance and susceptibility to tumor virus exposure. The usefulness and repeatability of this set

of crosses will increase with the uniformity of the lines. In any case, each mating will be made up of a random sample of the breeding stock of the lines involved.

The following kinds of information may be obtained from these matings in the study of a single trait:

1. The averages of the columns indicate the genetic contributions of each line when mated to all other lines.

2. The averages of the rows indicate the genetic and maternal contributions of each line when mated to all other lines.

3. A comparison of the average effects of the lines with the results of the specific crosses indicates whether specific combinations have special or interaction effects. That is, are the results of a particular cross predictable?

4. Reciprocal cross differences suggest non-genetic maternal or sex-linked genetic influence.

5. A comparison between the pure lines and crosses gives the effect of crossing on the trait.

The diallel cross illustrates the kind of information which may be obtained from a systematic experimental design. This type of approach indicates the existence of certain types of variables, but does not give any information on their mechanism of action.

Perhaps a more important use of this design is to provide a wide variety of genotypes to expose to different virus isolates by different methods. This may suggest procedures which are potentially useful in the testing of crosses or families for resistance to lymphomatosis. It is not enough to find an exposure method which can give a predictably high incidence of disease in susceptible birds. It must also do a good job of selecting genotypes which are resistant to natural exposure.

The above design can also be used to determine if genetic resistance to different virus strains or isolates is correlated or if resistance to other diseases is associated to resistance to neoplasms. Therefore, this design may contribute information on the specificity of genetic resistance in chickens, an important question from a practical as well as basic point of view.

A more fundamental phase of genetic study involves the question of the mechanism of gene action. Dr. Waters has presented three examples of very specific and striking effects of genes. They are: a single recessive gene for resistance to Rous sarcoma virus; a single dominant

gene for resistance to erythroblastosis produced by inoculation with high doses of RPL 12 virus; a cross showing extreme susceptibility to erythroblastosis and visceral lymphomatosis on inoculation with RPL 12 virus.

We now have available a variety of techniques for the basic study of genetic resistance. The combined use of tissue culture, embryos and hatched chicks provides almost unlimited flexibility in experimental techniques available for studying these large genetic differences. The following kinds of questions can be investigated. Is resistance manifested at the cellular level or is the intact chicken needed? Is genetic resistance due to inhibition of virus growth or to resistance to neoplastic change? Preliminary experiments conducted by Dr. Piraino indicate that the methods available in tissue culture can contribute materially to our understanding of the nature of genetic resistance.

We have at this Laboratory a unique combination of materials and methods available for the study of fundamental questions on the nature of genetic resistance to virus neoplasms.

Phase 2

Genetically uniform stock is the most suitable material for the study of non-genetic variables because genetic variation does not obscure experimental results. For this reason the genetics program of this Laboratory has concentrated on inbreeding. Each of the lines now have an inbreeding coefficient of over 95%. Theoretically this means that most of the genetic variation which existed within the lines 23 years ago has been eliminated. However, there is reason to believe that inbreeding in many species does not eliminate genetic variation as rapidly as simple theory predicts. Therefore, it is desirable to find actual measures of genetic variability in these lines. Blood typing is one method which has been used extensively in chickens, while skin grafting has been used particularly in inbred mice.

Dr. Okazaki and I have used the "graft vs. host" or Simonsen reaction in embryos in a preliminary study of variation within six of our lines. This reaction is thought to be an immunological reaction of adult donor cells against the foreign tissue antigens of the embryonic host (Simonsen, 1957). Runting of the embryo, massive spleen enlargement and lesions on the chorio-allantoic membrane indicate antigenic differences between donor and host. Our procedure was similar to that of Billingham and Silvers (1959) and was to collect whole blood from about five adult male donors of each line and inoculate each blood sample onto the chorio-allantoic membrane of about 10 - 12 day embryos of the same line and on 5 embryos of another line. Control embryos were inoculated with frozen blood or saline. Observations were made after six more days of incubation.

The results indicate that lines 7, 9 and 10 are relatively uniform for this trait, while line 151 shows some variability and lines 6 and 15 show extreme variability.

We plan to continue this work in pedigree matings and compare this reaction with the results of skin grafting. These preliminary results suggest a fairly simple genetic basis for the control of the major histocompatability differences influencing this reaction and agree with the work of Payne and Jaffe (1961) and Burnet and Burnet (1961).

In conclusion I would like to emphasize that the material and methods available at this Laboratory offer a unique opportunity for the basic and applied study of genetic disease resistance in chickens. I hope we will be able to pursue vigorously at least some of the work I have outlined.

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FIGURE 1

Line of Dam \ Line of Sire	6	7	9	15	
6	6	7 x 6	9 x 6	15 x 6	$\bar{X}_{D6}$
7	6 x 7	7	9 x 7	15 x 7	$\bar{X}_{D7}$
9	6 x 9	7 x 9	9	15 x 9	$\bar{X}_{D9}$
15	6 x 15	7 x 15	9 x 15	15	$\bar{X}_{D15}$
	$\bar{X}_{S6}$	$\bar{X}_{S7}$	$\bar{X}_{S9}$	$\bar{X}_{S15}$	$\bar{\bar{X}}$

Figure 1 - Diallel Cross. The text gives five kinds of information which can be obtained from this experimental design.

Cornell's Experience in Breeding for Resistance to Leukosis

R. K. Cole[1]/

This long-term project, as established by Dr. F. B. Hutt in 1935, had two primary objectives. The first was to determine the feasibility of controlling disease in poultry by selective breeding, with special emphasis on leukosis since this disease was then, as it is still now, of paramount importance to the poultry industry. The second objective was to combine such selection with selection for improvement in economic characters like egg numbers, egg weight, body weight, and efficient reproduction. I will say no more about this last objective except to point out that since considerable selection pressure was applied to these economic characters, less selection pressure remained for resistance to disease. Thus the rate and amount of progress in dealing with leukosis was obviously reduced, compared to that obtainable. That considerable success was obtained in the combined objectives is clearly evident from the performance of the Cornell Resistant stock during three years in the N. Y. R. Sample Test. The summary for the 4th, 5th, and 6th annual tests showed that the Cornell Resistant stock had, when compared to 24 other stocks then sold commercially in the Northeast, (a) the lowest mortality, (b) the fewest losses from leukosis, (c) a very satisfactory rate of lay of 208 eggs, compared to the highest of 212, and (d) a net profit of $2.40 which was second highest among the 25 stocks compared.

Selection of breeders has followed natural exposure to disease and has been based on individual performance, dam-family and sire-family averages, and on performance of progeny. We have continued to use proven breeders as long as they are capable of reasonable reproduction and, of course, as long as their progenies continue to excel in performance. The original plan envisioned the establishment of four stocks bred for resistance to disease (leukosis) and two bred for susceptibility. Among the former was a derivative from the relatively resistant families of the Cornell stock on hand in 1935, now known as the C-resistant strain. Females from these same families were crossed to imported males (Kimber strain) to provide the origin of the K-resistant strain. Two additional commercial stocks of proven performance were added at this time. The experimental plan called for simultaneous and similar selection for disease resistance in all four stocks and the subsequent crossing to yield a 4-way cross, in a manner which had been so successful in hybrid corn production. The families with the highest levels of mortality, especially that from leukosis, from among the original stock on hand

1/ Cornell University

in 1935, were used to establish the Susceptible strain. Actually there were to be two sub-lines of the susceptible stock. It was soon evident that the basic plan was too ambitious for the facilities available. Consequently the two susceptible lines were re-combined as one strain, and the two introduced stocks were discarded. The C-resistant, K-resistant, and Susceptible strains have remained closed since their establishment by matings made in 1936. The test reports are based on females only and cover the period of 43 to 500 days of age.

Natural Exposure:

We have relied on natural exposure to leukosis by brooding chicks near to adult birds and by employing a common caretaker for both the chicks and the hens. We have further used brooding facilities which experience had shown to be associated with fairly high levels of exposure, as indicated by subsequent mortality. Since the purpose of the testing was to identify differences in genetic resistance to leukosis, we have relied on natural exposure. We assumed that the basis for genetic resistance could lie in the integrity of the skin or mucous membranes and hence such an important mechanism of resistance should not be by-passed by injection.

Diagnoses:

For the most part, I have been responsible for the diagnoses, usually done on sick birds so that I can have the additional advantage of knowing the clinical symptoms of the disease. In all summaries, we have listed each mortality under just one cause, based upon considered opinion at the time of the autopsy. For example, a bird showing typical symptoms and pathology of neurolymphomatosis may, upon post mortem, also show lymphomatous involvement of the ovary. Such a case would be listed in our summaries as neurolymphomatosis. These ovarian tumors may occur as early as 7 to 8 weeks and would be present in 10 to 15% of the cases diagnosed as neurolymphomatosis. When in doubt, tissues are taken for histological study. We have also made it routine practice to section all neoplasms other than typical neuro- and visceral lymphomatosis.

Common Environment:

Within each year (population) all chicks, regardless of strain, have had identical environmental conditions from and including the period of incubation, throughout the brooding and rearing periods, and in the laying quarters. Although not necessarily constant nor uniform from year to year, or hatch to hatch, all chicks of a particular age-group have been given uniform conditions of environment.

Furthermore, the natural environment has not been modified by the use of disinfectants, drugs, antibiotics, or feed additives. Vaccination has been kept at a minimum and is now used only for bronchitis and there in a form that depends upon a natural mode of dissemination.

RESULTS

During the period of the last 15 years mortality from leukosis was only 2.9% in the K-resistant strain and 3.5% in the C-resistant strain. There were nearly 15,000 pullets in each strain. In both, the losses from visceral was somewhat greater than that from neurolymphomatosis, while losses from oculo were very few. In contrast, in more than 9,000 Susceptible strain birds the level of mortality from leukosis was 39.5% and neuro was several times as frequent as visceral lymphomatosis. The level of visceral, 5.7%, was about twice the total leukosis loss in the resistant strains and was, no doubt, a minimum because much of the neurolymphomatosis mortality came at an age prior to that at which the visceral form is likely to express itself. A few cases of erythroid and myeloid leukosis were found, and have been included in the over-all classification of visceral. These represented 2% of the cases of visceral lymphomatosis in the K-resistant and Susceptible strains, but nearly 8% in the C-resistant strain.

During the last 9 years losses from leukosis have been at minimum levels of 0.1 to 2.4% within both the K-resistant and C-resistant strains, so that this disease complex is no longer of much importance in these stocks. Only one death from oculo has been recorded during this 9-year period in the resistant strains. In earlier years, when losses were at a somewhat higher level, there was a tendency for a positive relationship between levels of neuro and visceral lymphomatosis.

In the Susceptible strain, the level of losses has varied from year to year, and has been extremely low in the last two years. The reasons for the variations are unknown but appear to be associated with different levels of exposure. The near-absence of leukosis in the last two years cannot be due to genetic changes. Already, in the new 1962 population, we are seeing a resumption of the experience of former years. Cases of neurolymphomatosis are occurring in chicks less than 7 weeks of age. It is too early to establish a definite pattern, but all evidence suggests that we can expect a level of leukosis in line with that experienced in previous years.

Total Mortality:

Mortality from all causes has, in the last 8 years, been quite satisfactory when one considers the duration of the test period (43 to 500 days of age), the management procedures employed in the experiment, and the fact that there is no culling. The K strain showed fairly high losses (27-30%) in 1955 and 1956 when it proved to be highly susceptible to a virulent NCD virus vaccine and to subsequent losses associated with CRD. Otherwise the two resistant strains showed total mortality from 8% to rarely more than 17%. Thus the low levels of leukosis mortality were not the consequence of mortality from other causes or incorrect diagnoses. I should also like to point out that the high levels of mortality (usually in excess of 50%, and up to 75%) in the Susceptible strain precluded more than a token selection for characteristics of economic importance.

Other Neoplasms:

During the last 15 years there have also been marked strain differences in frequency of neoplasms other than neuro, visceral, and oculo lymphomatosis. The levels in the K, C, and S strains were 0.20, 0.71, and 1.64% respectively. Two interesting observations have been made in respect to these neoplasms. Only approximately 25% of these neoplasms in the resistant strains were of connective tissue origin whereas in the Susceptible strain nearly 65% could be classified as fibromas, myxomas, fibrosarcomas, or as gliomas. Of the 30 gliomas recorded, 26 were in the Susceptible strain, which accounted for only 2% of the total bird population. These gliomas were similar to those reported by Dr. Douglas Coles of Onderstepoort, who found them to be associated with families experiencing high levels of neurolymphomatosis.

Among epithelial-cell neoplasms, the C-resistant strain was retatively susceptible, yielding 20 cases of adenocarcinoma while the other two strains combined had a total of only 3. This difference is accentuated in older birds from these strains, for adenocarcinoma of ovary, oviduct, and peritoneum is rather common in the C-strain hens kept for a second year of production.

Neoplastic Mortality in Strain Crosses:

Twelve years ago we crossed our two resistant strains and obtained good samples of purebred and crossbred offspring from the same sires. In these the level of leukosis was significantly higher among the crossbred than among the purebred progeny. Dr. Waters reported a similar trend in some of his crosses. However, when the strains were crossed again in 1959 the levels of losses in the pure lines were considerably lower than the earlier test and the

losses in the crosses were actually less than in the purebreds, being 1.4 vs 1.3 and 3.6 vs 2.8% for the two strains of sires used. That exposure had been high was obvious because the Susceptible strain had a loss from neoplasms of 47.1% in the same population.

Some years ago we also crossed the Resistant and Susceptible strains. Combined data for the two years of the test indicated that the losses from leukosis in the crosses were intermediate between that of the two parental strains. Such findings are in agreement with the hypothesis that resistance depends upon many genes.

Line 15 Females: (Leukosis-susceptible strain from the U. S. Regional Poultry Laboratory).

In 1954 samples of Line 15 chicks from the Federal Laboratory at East Lansing were hatched and raised with each of our pedigreed hatches. All told, there were 381 Line 15 females on which we have records. Mortality from neoplasms in this stock was only 3.8% compared to 52.8% in our Susceptible strain and to 0.8 and 2.3% in the K- and C-resistant strains. Losses were thus not appreciably higher than for our resistant strains, although the level of exposure was rather high. The Line 15 females did, however, suffer high mortality from other conditions such as intestinal parasites, respiratory infections, histomoniasis, cannibalism and miscellaneous causes. This reaction might have been expected, for Line 15 had not been subjected to artificial or natural selection against such common pathogens in the previous 15 years.

Relationship to General Disease Resistance:

No one stock has proven to excel in resistance to all diseases, and thus we may conclude that genetic resistance must be based in large part on resistance to specific pathogens or conditions. For example, the K-resistant stock is relatively susceptible to Newcastle disease, to respiratory infections, and to nephritis. In contrast, the C-resistant strain shows relatively more losses from avian monocytosis, bumblefoot, disorders of the reproductive system, and to a cystic condition of the right Mullerian duct. The Susceptible strain, which normally shows very high losses from leukosis, is also more apt to show losses from connective tissue tumors such as fibromas, fibrosarcomas, and gliomas. It is also subjected to internal laying of shelled eggs and to peculiar exostoses on the distal tarsometatarsi.

Other Studies on Leukosis:

Over the years we have tried a variety of experiments in an attempt to determine what environmental factors may influence the level of subsequent leukosis. These investigations have included the effect of (1) isolation of the young susceptible chicks, (2) restricted

feeding on range, (3) quality of diet during the early brooding period, (4) feeding cobalt, (5) crowding the chicks in the brooder, (6) ventilation during the early brooding period, (7) bursectomy, and (8) induced resistance to RPL 12 upon naturally occurring forms of leukosis.

Summary

Of all things tried, only selective breeding and isolation-rearing have yielded positive results, and both procedures, although not 100% effective, are still effective and are the best that we have to offer to the Poultry Industry.

Summary of Recent Achievements and Plans for the Future

B. R. Burmester

This Laboratory has been engaged in research specifically directed toward the development of possible control measures by three classical approaches, namely, (1) elimination of infection, (2) the use of vaccines, and (3) increasing natural resistance by genetic means. A fourth possible approach, i.e., the discovery of an effective therapeutic or prophylactic biochemical, has not received any attention here, largely because other government agencies have a very large program in this area.

Progress in all three approaches has been severely handicapped by the lack of good tools. Adequate tools, i.e. the methods, the procedures and instruments are one of the three basic components of good research progress.

Recently the situation has changed considerably. Dr. Rubin's discovery of the RIF test must be considered a distinct "breakthrough" in lymphomatosis research. To detect and at least roughly assay virus at levels that occur under natural conditions by a laboratory method is something that has never been done before. This test already has been of tremendous value and may well prove to be the key to progress by all three approaches mentioned.

At this point I should inject a note of caution. The RIF test will not do everything. First, the method itself, as you have seen, is long and cumbersome, requiring many manipulations. It is subject to many variables and quite expensive to conduct--as of now it can be used only as a research tool. It has many limitations, many of which I'm sure have not yet been determined. The overall specificity of the method has not yet been defined. Its sensitivity in relation to *in vivo* methods has not been fully determined. Some erratic results have been obtained; however, at the present time we cannot attribute them to the RIF method because of unknown variables and the lack of time to recheck the determinations. For instance, visceral and neural lymphomatosis have occurred in several pens of hens whose serums were found to be free of RIF virus and antibody a short time previously; also, a hen produced RIF free embryos for some time and then started producing resistant embryos.

Results obtained and experience gained in working with it in many different laboratories is required to determine its limitations as well as its potentialities. In this regard I am glad to say that a few experiment stations have already made plans to start leukosis research on this basis. Also, most of the manufacturers of biologics

are currently doing much work with this test because of the new standards for live virus vaccines. With so many experts working with the method, improvements are bound to be made and its limitations defined.

The RIF test will also lead to the development of other methods. Thus it provides a rapid means of checking reagents of specific viral or antibody activity which can then be used in other tests. The demonstrated close correlation between Rous virus antibody and lymphomatosis antibody means that we can use the Rous virus for at least field surveys of antibody occurrence. Dr. Sam Kenzy, in fact, has used this method for a number of years and his results become more significant with the continued studies on relation between sarcomas and visceral lymphomatosis. You have already heard about our progress with the complement fixation and fluorescent antibody tests. Neither one can yet be applied on a practical scale; however, both appear so promising that they may well supplement and in certain areas supplant the RIF test. Other types of tests also need to be reexamined for their possible adaptation to the reagents of this disease complex.

A reasonable test for virus and antibody is particularly important for research on lymphomatosis because so much of this disease remains inapparent. To develop its whys and wherefores is almost impossible when one is restricted to only the overt disease which may represent less than 1/10 of the entire picture. A familiar, similar example is avian encephalomyelitis. The studies by Dr. Rubin and Dr. Hughes reported yesterday is a good example of the kind of results that can be expected when the spread of infection can be followed with tests that detect virus and antibody.

Similar information is needed on other flocks of different breeding and environment before any kind of a control program can be intelligently formulated. Such questions as these must be answered:

> Can all or most flocks be divided into only two types--the viremic, and antibody?
>
> Is there an important segment that is free of infection and antibody?
>
> Is the proportion that are viremic, low, high, or variable?

With the availability of laboratory tests for virus and antibody, results obtained in eqizootiologic studies become much more meaningful. With this in mind, we have during the past year set up a cooperative

project to study all phases of the natural history and transmission of lymphomatosis. The agencies involved, in addition to this Laboratory, are:

1. Epizootiology Section of the National Cancer Institute.

2. Animal Disease and Eradication Division of Agricultural Research Service, U. S. Department of Agriculture.

3. American Poultry and Hatchery Federation.

4. Experimental, test, or farm flocks--owned by private or other agencies.

We not only plan to gather information relative to (1) prevalence of infection, antibody and neoplasms (2) modes, vectors, and reservoirs of infection, but also to set up controlled experiments in the field and laboratory to check the implications obtained by the data gathered, especially with respect to testing procedures or materials which may be helpful in reducing or eliminating high mortality.

At the present time there is quite a difference in opinion among various experts and others with regard to the relative importance of contaminated environment in the spread of infection and whether various fomites are instrumental in its spread. Many observations and claims have been made; however, there is still a lack of good data based on controlled experimentation. We hope that this question can be resolved in the near future.

The availability of a rapid test for virus and antibody should also make the road firmer and smoother for the geneticist. So far, he has had to work with an infected flock; moreover, he never knew what proportion of the flock was infected or had antibodies, or when the exposure took place and least of all, the amount or exposure dose. Even when he was prevailed upon to use artificial exposure, the result was often of doubtful interpretation because of the unknown prevalence of antibody.

Controlled exposure of progeny from infection-free antibody-free hens would be the obvious answer. Whether or not this is a feasible procedure remains to be determined. At least the tools are now available to check the antibody status of the dam so that passive immunity in the progeny may be eliminated as a complicating factor in the analysis of responses to experimental exposure.

Perhaps the greatest impact of the new laboratory methods will be on the development of a vaccine and procedures for its use. There is no doubt that the lymphomatosis virus is antigenic when given at the

right age to the right kind of individual; thus, chickens exposed when they are too young or as embryos may develop a tolerance to it and never develop antibodies; and older birds that are viremic have tolerant infections and will not respond to a vaccine.

There is some suggestion that the age of acquisition of immunological competence varies with the genetic constitution. The genetically resistant chick may acquire this earlier than the susceptible bird.

It has also been clearly shown that chicks with maternal antibodies are much more resistant to infection than chicks of the same stock but without antibodies, and more important, this resistance can be stimulated by injecting virus. A more continuing protection should be obtained if an active immunity is established after the decay in the passive immunity.

Based on currently available information, it would appear that a vaccination program may be successful; provided (1) the viremic, tolerant infected birds are eliminated from the flock either by testing or by simple dilution, (2) chicks are hatched only from immune hens which pass high levels of antibody to the chicks, and (3) at 3 to 8 weeks of age all chicks are vaccinated to produce an active immunity. If the latter is maintained during the laying year, the progeny should again have a passive immunity. In principle, the program would be very similar to that recommended for control of avian encephalomyelitis.

There are many difficulties that may arise; however, basic facts are now available and certainly point to an eventual development of a successful immunization procedure. Much of the success will depend on the use of the right vaccine. Our concern about this caused us a few years ago to hold in abeyance direct work on a vaccine, and do more work on the virus. We needed to examine more strains, especially those we could be quite sure represented the viruses in the field. We needed attenuated strains, and we needed strains which would retain high antigenic potency when infectivity was killed. Also, we needed to know something about the antigenic variation between different strains and isolates.

Actually, we don't know for sure what kind of a strain would make the best vaccine, let alone know which of the presently known strains would be the best. The isolation of a particularly good vaccine virus is largely one of chance. For this reason other laboratories should be encouraged to isolate and study new strains. There also should be a free exchange of strains from laboratory to laboratory. We have always followed the practice of freely sending virus upon request to anyone equipped to handle it. Each month we fill several requests.

You heard yesterday something of our effort during the past few years in the isolation and study of several new strains obtained from various parts of the country. The information on them is far from complete. However, they have been sufficiently well characterized to tell us that these 11 different strains, or some may want to call them "isolates", are all quite similar and in their oncogenic spectrum are no different than RPL 12 and other strains isolated from our genetic population. However, there is good indication of antigenic differences. Further research in this must be pushed. For this important aspect there must be frequent pooling of information and exchange of reagents between laboratories in order to make the most efficient and rapid progress.

We have again turned our attention to the more immediate problems of vaccine development, such as attenuation, inactivation, ages and times of vaccination, and antibody responses. We hope to be in a position to run preliminary field trials in the very near future.

I would like to leave this one thought that would stimulate research progress more than most people may think, and that is: That those of us actually engaged in research in this area should get together often for the sole purpose of discussing each other's problems, progress, and even "break-through." There is nothing more stimulating than a thorough going over of results and problems. With the increased interest stimulated by several factors, I hope and I fully expect several additional laboratories will take up research on this disease complex. It therefore becomes even more important and advantageous to all concerned that we get together at least once a year. There are many problems and pitfalls associated with research on this disease complex that are not evident to the novice.

I would like to say just one more word, and that is: in all the years that I have been at this Laboratory working on this problem, I have never been as optimistic as I am now concerning a solution in the near future.

Research Program in Avian Anatomy

Alfred M. Lucas

A program in basic studies on domesticated fowl was begun in 1944. It was planned to cover the subjects of gross, subgross and microscopic anatomy. We began with that which seemed most important; namely the description of avian blood cells and the developmental stages for each cell line. When we began, we didn't realize the extent of the task before us and we thought that there would be ample literature on the subject. Instead, it was soon discovered that we were faced with one big research problem from beginning to end.

In hematology there is a common language throughout the world; namely, good pictures. We tried all of the short cuts, including photographs but none of them were adequate. There is no known substitute for colored illustrations made by a competent artist. Of course, it takes time and it costs money, but a job well-done in basic research does not have to be redone the next week, the next year, or the next decade.

After some 11 years of study and writing, aided by the artistic talents of Casimir Jamroz for 4 years, the Atlas of Avian Hematology was completed in 1955. The book came off the press in 1961. I would like to show you a few of Mr. Jamroz's fine illustrations. These will indicate the scope of the work. A photograph of an actual slide from which a drawing was made is included so that you may judge for yourself if photographs would have done the job.

Since this is a basic study it should serve as a new tool for many disciplines: veterinary medicine, pathology, physiology, poultry research, experimental morphology, embryology, zoology, ornithology, blood parasitology, wild life research and others.

It is not the function of this book to tell what happens when you inject a virus into an embryo. Rather, the function is to provide a description of each kind of cell that the investigator is likely to find in any experiment. A second function is to provide a standardization of terminology in the hope that repetition of similar experiments done by several investigators will not lead to seemingly contradictory results, merely because their ideas of terminology are different.

The Atlas is being reviewed in 85 journals of the world, representing 24 countries. In addition to the United States, these countries are: Germany, Great Britain, India, France, Netherlands, Israel, Australia, South Africa, Austria, Belgium, Japan, Switzerland, Argentina, Canada, Ceylon, Chile, Denmark, Greece, Indonesia, Italy and Mexico. The only country behind the Iron Curtain is Yugoslavia.

I believe everyone who does basic research receives a tremendous reward when he finds a utilization for his product, and now I would like to give one example of the practical application that grew out of the Atlas. This opportunity came to us about 3 months after the book was published. Dr. G. S. McKee of Agricultural Marketing Service reported that turkeys as seen on the processing line showed lesions in the spleen suggestive of leukosis. If this should prove to be true there would be large losses to the turkey industry. The regulations under which the inspector operates, state that if there is a leukotic lesion in the viscera, the whole bird is to be condemned. Representatives of AMS and ARS went to the processing plants, collected turkey tissues from the inspectors' line, made sections and came up with an answer to which the earlier basic studies in hematology provided the guide lines. The answer briefly was this: although leukosis does exist in turkeys, the leukotic-like lesions commonly seen in the spleens represent merely tissue reactivity to arrested chronic cases of blackhead. The conclusions from this relatively simple study have repaid to the public the cost of the Atlas several times over.

About 6 years ago when our work on the Atlas had been completed, Mr. Winton approached me with the request that we prepare an authoritative series of monographs on the gross and microscopic anatomy of the fowl. I insisted at that time that he probably had no concept of the magnitude of the task and probably now he, as well as our administrative group in Washington, will agree that the size of the job far exceeds anyone's anticipations. But, the job is started. Progress toward the first volume has been made and with adequate support we will finish the task, but let me tell you something about the scope of the work.

There are 10 organ-systems of the body: integumentary, skeletal, muscular, vascular, nervous, digestive, respiratory, excretory, reproductive and endocrine. During these 6 years, progress has been made on some of these systems, and samples of what has been accomplished are shown on the display panels at the back of this room. Dr. Stettenheim will review some of the new data that has been collected and during your tour of the Laboratory there will be additional items for your examination.

Gross anatomy alone is not enough--histology is included in these studies because it is particularly useful to the pathologist, the physiologist, the pharmocologist and, in fact, everyone who studies host reactions to stimuli.

We can only review in broad terms what has been accomplished thus far. It can be stated categorically that we move forward only as fast as we have available artists to prepare illustrations. We cannot prepare a manuscript until the drawings are finished, because the text and figures supplement each other so closely.

The following topics on the integumentary system are those on which we feel that considerable progress has been made: (1) establishment of boundary areas for each region of the avian body, (2) a detailed study of the feather tracts and non-feather areas, (3) the microscopic structure of various parts of feathers from different regions of the body, (4) the sequence of molting for each feather of the body up to 6 months of age, (5) the distribution, histology, development and molting of filoplumes and (6) the morphology and histology of the smooth muscles that move the feathers.

Most of this applies only to the chicken and certain phases will be extended to other domesticated birds, namely, turkey, duck, pigeon and Japanese quail.

We have yet to work out blood supply and nerve supply to the feather muscles, the skin and specialized structures of the skin and we have a great deal to do on the histology. Thus far only two drawings on this subject have been completed. These picture the growing feather in its follicle and when this series is done, we still have to illustrate the histology of unfeathered skin, claws, scales, ear lobes, comb, wattles and so on. Some of these will be in the form of diagrams showing histologic structures in three planes. Such schematic diagrams usually reduce the number of cross section illustrations that are needed.

The first volume of this series of monographs will cover only the integument. It is hoped to limit the size of each volume to about 300 pages and it is not possible to say exactly how many organ-systems can be covered in each volume, although we do know the order in which the various subjects will receive consideration.

A tremendous amount of technical work is needed throughout these studies. I would like to mention a staff member who has so effectively carried out the supervision of this phase of the work. Mrs. Effie Denington has prepared slides when we needed them and her preparations in histology, neurology and hematology are beautiful to behold in the microscope. Nothing that she does can be considered routine. Each day's needs differ from those of the day before and from the day ahead.

The next topic is the muscular system. Through the efforts of part-time veterinary students the basic dissections and study have been completed for body, tail, leg, wing, neck and most of the head. The origins, insertions and fiber direction for each muscle have been described and drawings of the bones of the body have been completed to show muscle attachments. A few samples of the work sketches as they now stand are displayed on the panels at the rear of the room; some 50 drawings were made to show the muscles of the wing and about 45 for the leg.

Practically all of the bones of the chicken's skeletal system have been photographed, often from 4 to 6 directions to show all surfaces and facets. This is a temporary makeshift to which we can add working labels. Eventually drawings will be made. A drawing shows more of the important details than even a good photograph.

We have yet to check other species of domesticated fowl in order to identify differences and similarities in relation to the chicken. Nothing as yet has been done with the ligaments that bind the bones together. The muscular and skeletal systems cannot be considered completed until the vascular system and nervous systems have been traced to all parts of the body. As you see no one part of the body is isolated from all other parts.

An analysis of the internal structure of the avian brain is a full-time job for at least one to two years. We cannot by-pass or ignore the task--the physiologists at present are putting electrodes into the chicken brain and they have very little to guide them on the internal architecture of the brain. The pathologist is interested also. A short time ago I had a letter from the person in charge of the diagnostic laboratories in Pennsylvania asking where he could find a description of the internal structure of the avian brain. We sent a list of several references but actually none of them gave him the kind of information he needed.

The respiratory system is the only visceral system that has received any study to date. Soon after we began these studies, a practical application developed. Dr. G. S. McKee invited me to present this material at training courses for area and technical supervisors of the inspection service.

Then mandatory inspection went into effect and air sac infection was a significant part of condemnation. Regional meetings were held in southern and eastern parts of the country and we were frequently invited to present the basic anatomy of the respiratory system in order to provide a common understanding in the discussion of disease problems and in the reasoning behind the regulations.

Numerous requests came to the Laboratory for duplicate sets of the lantern slides. These were handled through a local processor and to date he has supplied 71 sets to various university departments, to federal and state governments and to commercial organizations. Recently, a chick incubator company reproduced the color illustrations of the turkey as part of a 4-page brochure.

When so many drawings are needed and each must be exact in every detail, we were forced to invent a drawing machine in order to speed production. All photographs introduce distortion due to perspective. The drawing machine gives us orthographic projection as employed in making mechanical drawings; a drawing of an inch square far from the viewer is the same size as an inch square close to the viewer. Well over a hundred drawings have been made with the machine thus far. On your visit to the Laboratory the apparatus and its operation will be available for your inspection.

In closing will you step into our shoes for a moment? From the middle of the last century to the present, thousands, even tens of thousands of publications have appeared in all the scientific languages of the world describing the anatomy and histology of man and mammals. When in 1956 we began the Anatomy Project at Mr. Winton's request, we were undertaking almost single handed to match in coverage and detail for domesticated birds what nearly a century of study had contributed to the knowledge of man and mammals.

Can we be less thorough? I think not. A chicken may not have the economic or social status of a man but to really understand disease problems in either species requires the same equivalent backlog of scientific knowledge. In poultry, as in other branches of science, the day of empirical research is fast approaching the point of diminishing returns and we are faced now with building our sciences upon a solid foundation of detailed anatomy whether we like it or not. Our discrimination of the pathological can proceed only as fast as we clearly describe the normal.

Studies of Feathers and Integument

Peter Stettenheim

Although a chicken appears to be entirely clothed in feathers, the feathers are not distributed uniformly over its body. They grow in tracts (pterylae) separated by areas of few or no feathers (apteria). The arrangement of the feathered and the unfeathered areas is distinctive for most species of birds, and hence has been used as a taxonomic character. We have worked out in detail the distribution of feathers on chickens, pigeons, and Coturnix quail; we plan eventually to do the domestic duck and turkey also. While the poultryman's names for feathered areas are sufficient for his needs they cannot be applied to the tracts. Still less can terms for a chicken be used for other domesticated birds. Our terminology, therefore, is based on that used by ornithologists.

The 16 major feather tracts of a chicken are as follows:

capital	caudal	femoral
dorsal cervical	lateral	crural
interscapular	ventral cervical	humeral
dorsal	pectoral	dorsal alar
pelvic	sternal	ventral alar
postpelvic	abdominal	

Some of these, such as the capital and alar tracts can be subdivided.

Feathers vary widely in size, shape, and pattern over the body. In order to show this variation we prepared ten large panels such as the one exhibited in the hallway. To make each panel, we plucked the feathers from a region of a chicken and marked every empty follicle with a dot of ink. This area of skin was then cut out and photographed. The image of the negative was projected onto a 4 x 8 foot panel, and the locations of the follicles marked with white dots. Finally, we took a fresh chicken, plucked the same area, and glued its feathers onto the corresponding follicle dots. The result was an "exploded" view of the tract, an unusually effective type of presentation.

All intergrades can be found among feathers, even those as different as the tiny ones on the face and the long ones on the back. This has led us to examine and revise the criteria commonly used for distinguishing the major structural types--contour feathers, semiplumes, down feathers, filoplumes, and other special types.

A contour feather, the commonest type, consists of a shaft bearing approximately 150 to 300 branches or barbs on each side. There is usually a small feather-like structure, the after-shaft, attached to the underside of the shaft at the level of the lowest barbs.

The distinctive feature of a contour feather is that at least some of its barbs hold closely together. Each barb bears a few dozen pairs of tiny branches, the barbules. The character of the barbules determines the texture, and ultimately the structural (as distinguished from the pigmented) pattern of a feather. The closely-knit portion of the vane is produced by the interlocking of two kinds of barbules. Those on the distal side of one barb have hooklets and cilla which engage the flanges on the barbules on the proximal side of the next barb. The loose, downy portion at the base of the vane is composed of barbules which cannot interlock, owing to the absence of hooklets and flanges. The character of barbules changes along the length of each barb, and among barbs from the base to the tip of the shaft. A thorough study of feather structure thus can be a complicated job.

Semiplumes are loose-webbed feathers in which the shaft is longer than the longest barb. They commonly have aftershafts. Feathers of this type occur along the borders of the cervical, pelvic, and abdominal tracts. Down feathers are distinguished by having a shaft which is shorter than the longest barb. Aftershafts are present on adult downs but not on natal ones.

The hairlike structures which remain after a chicken has been plucked are called filoplumes. Very little has been reported about these modified feathers, so we carried out a study of them last year. Filoplumes grow on most of the feather tracts, in varying degrees of abundance. They are always situated close beside regular feathers, and these must be of the contour or semiplume type. In most cases there are but one or two filoplumes per feather, but around each of the remiges and rectrices there may be as many as eight. The filoplume follicles form during embryonic life but the filoplumes themselves do not appear above the skin until the second generation of feathers appears. From then on, they molt at approximately the same time as the feathers with which they are associated.

A filoplume grows in basically the same way as a regular feather, and homologies between them are revealed in the developmental stages. The distinctive feature of filoplume growth is that only a few barbs unite with the shaft. The remaining barbs form but they are lost soon after the filoplume emerges from its sheath.

Let us now return to the typical contour feather for a look at its development. The spatial relationship of the parts of a young feather is the key to an understanding of the structure of a mature feather. Hence, although embryology is beyond our scope, we are preparing illustrations of growing feathers. If you have looked closely at a feather just emerging from its sheath, you know that in its growing stage the feather is rolled into a tube. Blood vessels and pulp fill the center of the tube and a thin sheath of

keratinized epithelium encloses it. The parts of the feather itself arise from a ring of epidermis at the base of the follicle. The barbs and barbules do not grow out from the shaft like the branches of a tree. They form ridges which grow at such an angle that they eventually join the shaft. Their development proceeds as they move upward in the follicle.

The formation and molting of plumages have been studied a great deal by poultry scientists and others. Nevertheless, the sequence of feather growth within and among all tracts had not been followed in detail. We investigated this subject by making repeated observations on a group of chicks from hatching until six months of age. During this time a chicken produces four generations of feathers (plumages), counting the natal down as the first.

In our birds the first molt began soon after hatching, the second about the 6th week, and the third about the 19th week. The second and third molts each start before the preceding molt is finished. Feathers are consequently growing and being replaced somewhere on the body throughout at least the first six months. The feathering always includes at least two plumages, though its composition is constantly changing. Molting follows a definite sequence both within and among tracts. This order is established in the embryo and repeated with diminishing precision at each of the first 3 molts. Within each tract there is a focal area where molt and development begin, and from which they spread.

Body structures cannot be properly understood if they are studied only as separate entities. Each must be considered in relation to its blood supply, innervation, muscles, and so forth. These topics have also been included in our studies of feathers. Two kinds of muscles move feathers--(1) sheets of striated muscle which are the dermal components of certain body muscles, and (2) tiny bands of non-striated muscle which interconnect adjacent follicles. These muscles attach to the follicles, not directly to the feathers. The striated muscles produce major movements of the skin and groups of feathers. The non-striated, or "feather" muscles raise and lower individual feathers or draw them closer together. There are no circular muscles around the follicles.

Basically, each contour or semiplume feather is connected by pairs of muscles to each of the four feathers nearest it. The size and number of the muscle bands varies over the body in relation to differences in the extent of movement in the feathers. As you have seen a rooster raise its hackles, it should not surprise you that the feather muscles are heavily developed on the neck. They are weakly developed in such places as the inner surface of the legs.

The nerve supply to the skin has two elements currently of interest to us. First are the nerves to the dermal and the feather muscles, just as in other muscles. Second are the Herbst corpuscles, nerve endings which resemble the Vater-Pacini corpuscles of mammals. These structures have been found in various parts of the body but in the skin they are located only near feather follicles. They have been shown to be receptors for vibration, and may play a role in the positioning of the feathers.

Our research program has been directed toward the gathering and publication of basic information on avian anatomy. The information does have application, however, as shown by the many requests we have received for help on specific problems. The formation of feather follicles in the embryo was of concern to a student working on piebald spotting patterns. The structure and molting of certain feathers in fancy breeds of chickens have interested men who utilize these feathers in trout flies. Knowledge of the microscopic structure of barbules enabled us to identify feathers which had been taken as clues in criminal cases. We have discussed the feather muscles with Dr. Robert Ringer of Michigan State University, who is investigating methods for releasing a feather from its follicle.

Many requests for information have come from physiologists, poultry scientists, and veterinarians. When these concern parts of the body we haven't yet given much attention, we sometimes can't give very good answers. We hope eventually to produce a series of reference books that will do so.